THE VASODILATOR AND VASOCONSTRICTOR PROPERTIES OF BLOOD SERUM AND PLASMA.*

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PLATE 8.

The introduction of the Loewen-Trendelenburg (1) frog preparation for the quantitative and qualitative determination of suprarenin aroused high hopes that at last a method had been discovered whereby the internal secretion of the adrenal glands could be estimated in both physiological and pathological conditions. The Ehrmann-Meltzer (2) frog's pupil preparation is not sensitive enough to detect small variations in the concentration of suprarenin in the blood; the ox carotid method of Meyer possesses the disadvantage that its application in the case of foreign sera is untrustworthy (Schlayer (3)); the virgin uterus of the rabbit may react by either contraction or dilatation (Falta and Fleming (4)); while, finally, the inhibition produced on a strip of intestine is so uncertain that this method alone is of small value.

Toward solutions of pure suprarenin the Loewen-Trendelenburg method is intensely sensitive. It will detect with ease as small a oncentration as one in forty million. It is unquestionably the most accurate method for the quantitative determination of solutions of pure suprarenin. Moreover, the test is easily applied, and very small amounts of the solution are necessary. But it has been found that when used for a fluid as complex as blood serum, it is not specific for suprarenin. Its use may, however, be said to have led to the discovery of a new class of vasoconstrictor substances in blood serum.

The observation was made by O'Connor (5) and confirmed later by Falta and Fleming, that the quantitative estimation of suprarenin

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in serum, obtained by the Loewen-Trendelenburg preparation, is at variance with that given by the uterus preparation. The latter as compared with the former gave results that indicated a higher concentration of suprarenin. O'Connor therefore concluded that the discrepancy arose from the presence of an additional vasoconstrictor substance and that the uterus preparation recorded the combined action of this substance and the suprarenin. He was able to show that this newly discovered constrictor substance is not found in an active form in plasma, but is liberated during the process of coagulation.

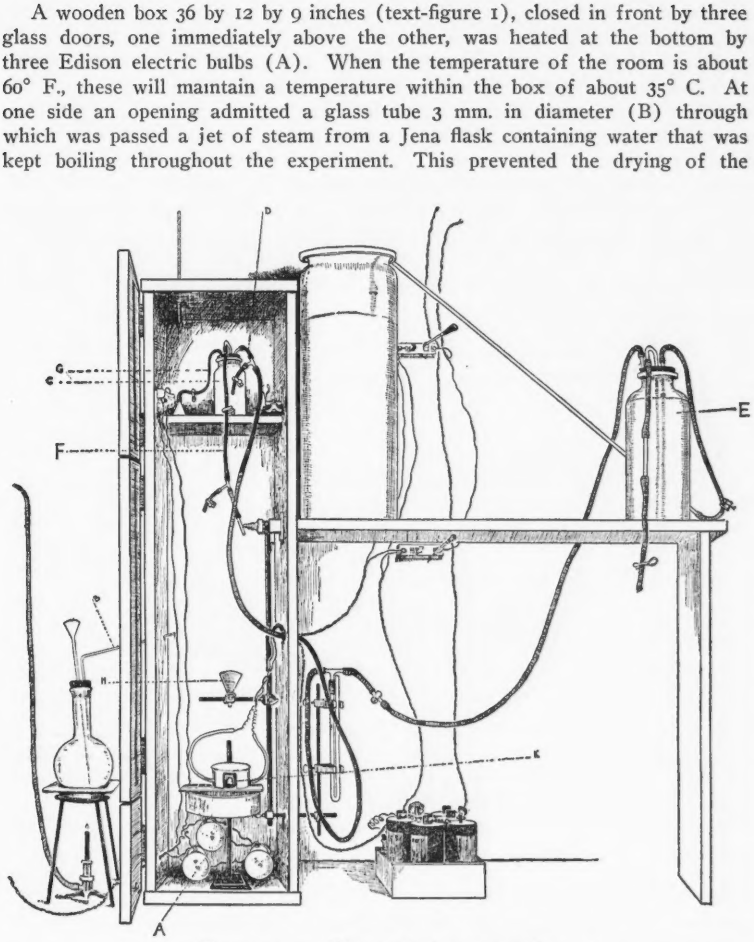
While O'Connor's work was still unknown to us, we observed that the blood serum from a patient with Addison's disease possessed as great a constricting power on the vessels of the isolated rabbit kidney as did that of normal serum, although after the death of the patient no suprarenin could be extracted from the remains of the suprarenal gland. We interpreted this result at the time as an indication of the presence of a vasoconstrictor substance other than suprarenin in the serum of the patient with Addison's disease. We determined, therefore, to make a systematic study of the vasoconstrictor action of the serum in this disease, and we began by establishing a standard in normal serum. We have observed, however, some hitherto undescribed properties of the sera of normal animals and these observations form the basis of the present communication.

METHODS OF INVESTIGATION.

Profiting by the experience of Schlayer that foreign sera are less sensitive on a strip of artery than homologous sera, we at first so arranged our experiments that the properties of serum were tested upon the vessels of an animal of the same species. The vessels employed were those of the kidney, the hind limb, and the heart of the rabbit, dog, and cat. The principle of the apparatus is essentially that used by Trendelenburg; namely, the determination of the rate of flow of fluid perfused through the isolated organs. Since we are dealing, however, with mammalian organs, perfusion had to be carried out at the temperature approximating that of the body, and in a moist atmosphere so as to prevent drying.

We found the following device very convenient and satisfactory.

A wooden box 36 by 12 by 9 inches (text-figure 1), closed in front by three glass doors, one immediately above the other, was heated at the bottom by three Edison electric bulbs (A). When the temperature of the room is about 60° F., these will maintain a temperature within the box of about 35° C. At one side an opening admitted a glass tube 3 mm. in diameter (B) through which was passed a jet of steam from a Jena flask containing water that was kept boiling throughout the experiment. This prevented the drying of the

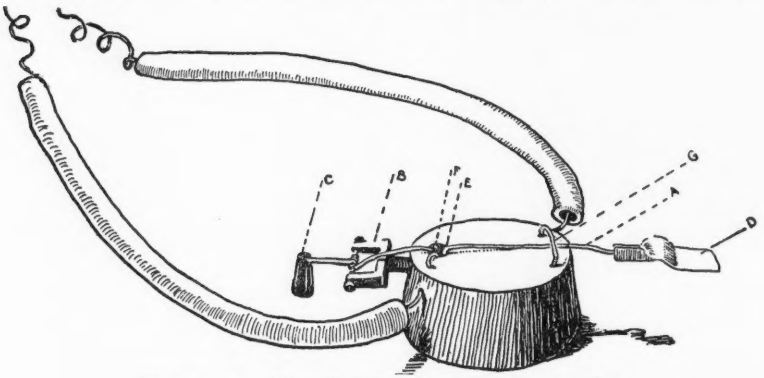


TEXT-FIG. 1. The perfusion apparatus.

perfused organ and supplemented the electric bulbs in keeping the temperature at about 37° C. A small shelf was inserted inside the box ten inches from the top, on which rested a thick glass bottle containing Ringer's solution (C). Through the cork stopper of this bottle three holes were bored, and each admitted glass tubes. One tube (D), which just passed through the cork, was connected with a pressure bottle (E); a second tube (F) served as the outflow and was connected by rubber tubing with the cannula inserted in the artery;

and the third tube (G), connected with a rubber tube clamped at the opposite end, served as a means for filling the reservoir. After passing through the organ, the perfusion fluid was allowed to run down the side of a filter funnel (H) and the rate of flow was determined by a drop decorder (K).

We tried many forms of drop recorder, but the most satisfactory, in that it responded to a frequency of drops just short of a constant stream, is that depicted in text-figure 2. It consists of a large rubber cork carrying a platinum wire (A). For this purpose the wire of the ordinary bacteriological platinum loop answers perfectly. Toward one end the wire passes through the pivot (B), and at (C) is attached a weight of about 5 gm. The opposite end carries a flattened piece of aluminum (D) upon which the drops fall. A small loop of the wire (E) is continuously immersed in a well of mercury (F) which is connected



TEXT-FIG. 2. Magnified drawing of drop recorder.

by a wire running to a terminal of the battery. Contact is made against a platinum cross wire (G). A constant current runs from three dry batteries, the circuit being broken by each drop. The use of platinum is necessary to prevent rusting of the contacts in a moist atmosphere.

After a few experiments we discarded the use of a cannula inserted in the vein, as we found that the least movement caused kinking and very seriously interfered with a constant rate of outflow. It was much simpler and much more accurate to allow the fluid to trickle from the vein into the funnel. Perfusion was maintained under a constant pressure of 40 mm. of mercury.

The Ringer's solution used had the following composition: sodium chlorid, 0.9 per cent.; potassium chlorid, 0.035 per cent.; calcium chlorid, 0.024 per cent.; sodium bicarbonate, 0.03 per cent. While the organ was being perfused with Ringer's solution, the serum to be tested by a hypodermic syringe was injected through the rubber tubing that conveyed the Ringer's solution to the organ. This method has the apparent objection that the serum is diluted by the perfusion fluid and does not reach the organ in a concentrated state. We found, however, that this objection is more apparent than real, for the rubber tubing is so narrow that the amount of serum used for each test, usually 2 c.c., when injected rapidly, drives the Ringer's solution backwards and reaches the organ before any great dilution has taken place. The drops were recorded

by a signal magnet on a smoked drum on which also a Jacquet chronograph marked off the time in seconds (figure 1).

The experiments may be divided into two main groups; those that deal with the effects of serum and plasma (1) upon the renal vessels of the dog and rabbit, and (2) on the peripheral vessels of the rabbit and frog. Human, dog, rabbit, and sheep sera were tested.

In a few experiments the action on coronary arteries was tested by perfusion of the heart of the rabbit. In these experiments we used a smaller pressure in order to prevent the organ from beating. Injections were made as in the case of the kidney and limb.

EXPERIMENTS ON THE KIDNEY VESSELS.

Three distinct phases are observed when the kidney is perfused with Ringer's solution. There is a preliminary increase in rate which rises in a few minutes to a maximum while the organ is being washed free of blood. The first phase shows a fairly rapid rate of outflow and persists from seven to twelve minutes. The number of drops of outflow per minute then gradually diminishes until it is less than half that of the original rate. This period of diminishing outflow, or second phase, varies with different kidneys, and even with the kidneys of the same animal. It may last from fifteen minutes to one hour, but it is always succeeded by the third phase, a period during which the rate of outflow is constant. We have endeavored in all of our experiments to inject the plasma or serum to be tested only when the rate of perfusion had reached this period of constancy.

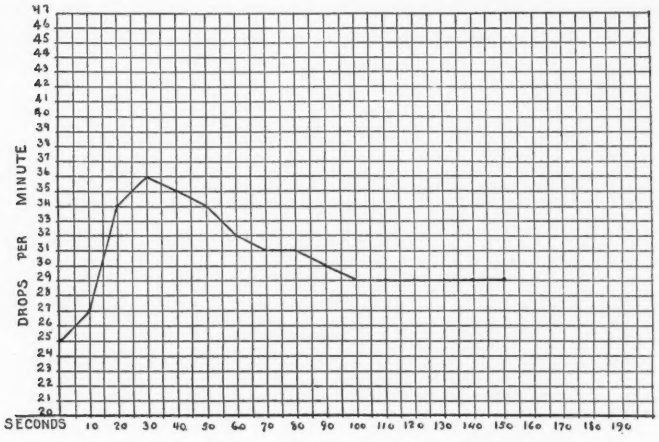
Fortunately, as far as the test is concerned, the effect of serum on the kidney shows itself as soon as it has reached the arterioles and lasts for a short period of time only. This stands in contrast to that which is observed in the vessels of all the other organs tested. In the case of the peripheral and coronary vessels, it sometimes takes half an hour for the outflow to return to its original level.

While the above description holds for the majority of experiments, it is to be emphasized that great variations occur and that no two kidneys exhibit phases of exactly the same duration. However, we have never failed to observe a period of constant flow, and

when tests are made within the limits of this phase, very clear cut and delicate reactions may be obtained.

In our earlier experiments the changes in ureter flow were recorded in addition to the outflow from the vein. Since, however, we soon noticed that the flow from the ureter was the same under all observed conditions we confined our subsequent observations to the venous outflow.

Text-figure 3 is a composite curve obtained from the venous flow



TEXT-FIG. 3. Effect of dog serum on rabbit kidney.

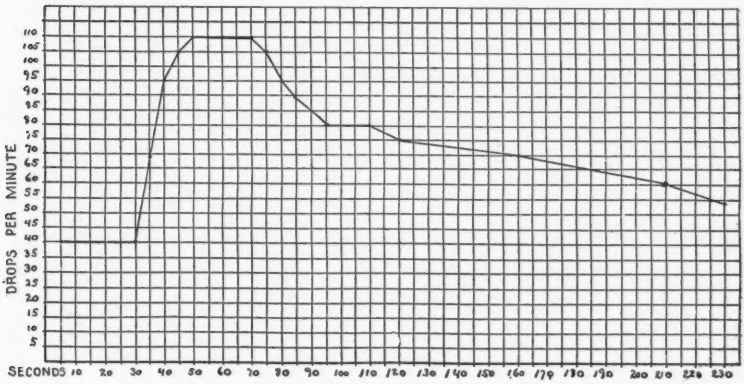
through rabbit kidneys after injections of dog serum. The abscissæ represent time in seconds, and the ordinates the change in the number of drops of outflow from the vein per minute.

The essential effect produced by the injection of two cubic centimeters of serum into an excised kidney perfused with Ringer's solution is, first, a rapid increase in the velocity of outflow, amounting in many cases to 150 per cent. The reaction begins as soon as the serum enters the kidney vessels, and gradually reaches a maximum which is attained in from twenty to thirty seconds. The maximum rate of outflow is maintained for only a few seconds. In fact, in many experiments the curve begins to recede immediately after the highest point is reached. The return to the original rate is much more gradual and not infrequently shows an irregularity for which

it is difficult to find an explanation. The fall in rate after the maximum dilatation is rapid during the first twenty seconds. Thereafter, the flow is much more gradual. A point slightly in excess of the original rate is reached usually in about two minutes. It is not unusual to find that this slightly increased rate may persist for several minutes before the normal outflow occurs. We are inclined to attach some importance to the fact that after the maximum has been reached the rate with which the curve falls to normal is faster at first than it is later, and to this we shall have occasion to refer in another part of this paper.

Figure 1 is a representative tracing which is charted in text-figure 4.

The ureter outflow shows a fall coincident with the arterial dila-



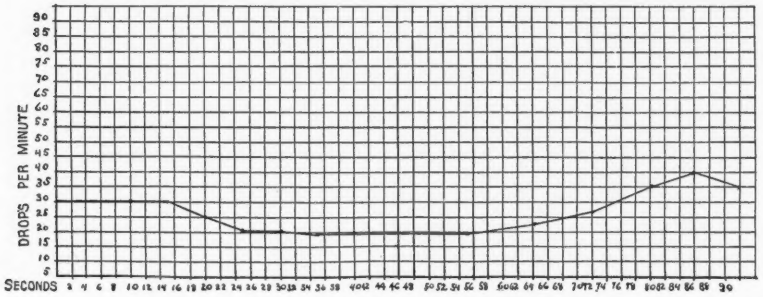
TEXT-FIG. 4. Effect of dog serum on rabbit kidney.

tation, but in contrast to the venous outflow, recovery is much delayed and frequently the outflow ceases entirely. We have not been able to determine the factors that influence ureter flow even when the kidney is perfused with Ringer's solution alone, for we have seldom been able to obtain a constant rate for any length of time. After the injection of serum into a kidney, the flow through the ureter rarely returns to normal.

The sera of different animals vary markedly in their vasodilator effect on the vessels of the rabbit kidney. Of those tested, we have found that the serum of the dog acting either on dog or on

rabbit kidney is always the most efficient in causing vasodilation. It causes a much more abrupt response than do other sera, and the increased outflow frequently amounts to three times the original. Return to normal is more gradual than with rabbit serum, but otherwise the sera are essentially similar in their mode of action. Human serum also possesses a vasodilator action which varies in different individuals, but is, as a rule, less potent than the serum of the dog or the rabbit. Sheep serum is the least active of those we have examined.

While in the majority of experiments rabbit serum when tested on rabbit kidney reacts in the typical manner already described, we have observed in eight experiments out of a total of thirty-four that the reverse result may be obtained; namely, either a vasoconstriction alone or a vasoconstriction followed by a vasodilatation. An example is seen in text-figure 5 (experiment 28). In this figure the



TEXT-FIG. 5. Effect of rabbit serum on rabbit kidney.

injection of two cubic centimeters of rabbit serum caused a fall in outflow amounting to 45 per cent. and was followed in the course of a minute by an increased outflow of 22 per cent.

In another experiment (experiment 20) the diminished outflow was the dominant feature and was not followed subsequently by an increased outflow.

The reversed action is to be attributed to the state of the kidney rather than to the serum, for the same serum tested on dog kidney produces the usual vasodilator effect. Unfortunately, the kidneys that did not react in the usual manner to serum were not examined histologically for the presence of pathological changes.

The kidney of the rabbit alone exhibits this reversal. We have not observed it in the dog or cat.

Repeated injections of serum tend to establish a condition of insensitiveness of the kidney vessels. It is probable that this is the result of a partial exhaustion of the vasodilator mechanism, since a kidney rendered inactive by repeated injections of rabbit serum will not respond further to dog or human serum.

To all the sera tested the kidney of the dog reacts in identically the same manner as does that of the rabbit except that no instance of reversed action has been observed. Nevertheless, we have found dog kidney less sensitive as a routine test for the vasodilator substance, because it very readily becomes edematous and soon ceases to respond to vasomotor influences.

It may be mentioned at this point that the vasodilator power of serum is specific for the renal vessels. When serum is tested on the peripheral vessels of both warm and cold blooded animals, the opposite effect, namely, a pronounced vasoconstriction, is always produced. Separate constituents of the blood serum are responsible for the different effects on the two sets of vessels, and the causal factor cannot be ascribed to one substance acting differently on different arteries.

As far as we have seen, the first work in the literature dealing with the vasomotor effect of blood serum is that of Brodie which appeared in 1900, although it must be mentioned that Stevens and Lee (6) had previously encountered great difficulty in perfusing the frog and terrapin with defibrinated blood. Brodie (7) found that the injection of blood serum, from whatever source, into the external jugular vein of a cat caused an immediate depression of the blood pressure. In addition there was also produced cardiac and respiratory inhibition. He gives clear proof that the fall in blood pressure is not explained by the cardiac inhibition alone. Thus the fall can occur in some animals in which there may be no noticeable inhibition, and section of the cardiac branches of the vagus does not prevent its occurrence. By plethysmographic records he also eliminated the possibility of a direct action of the serum on the heart muscle. The effect is almost entirely a reflex through the vasomotor center from excitation of the pulmonary nerves.

Although in the cat the general effect is a dilatation of the systemic arterioles, the kidney forms an exception. According to Brodie, the injection of serum in the cat causes a constriction of the renal vessels. This conclusion, however, is probably erroneous. Brodie considers the action on the kidney to be due to a reflex stimulation of the vasomotor center for the vessels supplying the kidney. Since experiments on the dog and rabbit were negative, it would seem that the cat is possibly the only animal which reacts in this manner to injections of serum.

While Brodie's experiments deal essentially with a substance acting by reflex inhibition of the vasomotor center and in that respect differ from the effects we have described on the isolated organ, he must be given the credit for having given the first demonstration of the fact that serum and plasma are not identical in their physiological activity. Since the publication of O'Connor's work, to which reference has already been made, this difference has received much attention. Brodie discovered the following facts concerning the nature of the substance that acts through the vasomotor center: first, that it is a proteid of the albumin class, coagulable by boiling, and formed during the process of clotting; and, secondly, that the corpuscles take an active part in its formation.

We have not repeated Brodie's experiments, but on the isolated cat kidney we have found that serum produces a pronounced vasodilatation and that the kidney of this animal differs in no way from that of the rabbit and dog.

Comparing Brodie's experiments with our own it would seem difficult at first sight to reconcile the facts that serum acting peripherally causes a dilatation of the kidney vessels, but acting centrally leads to a constriction of these vessels through a reflex stimulation of that part of the vasomotor center that supplies the kidney. It might of course be argued that two distinct substances are present in the serum, each having its selective action and affinity. It is, however, unnecessary to make this assumption in view of the work of Sollmann (8) which appeared six years later.

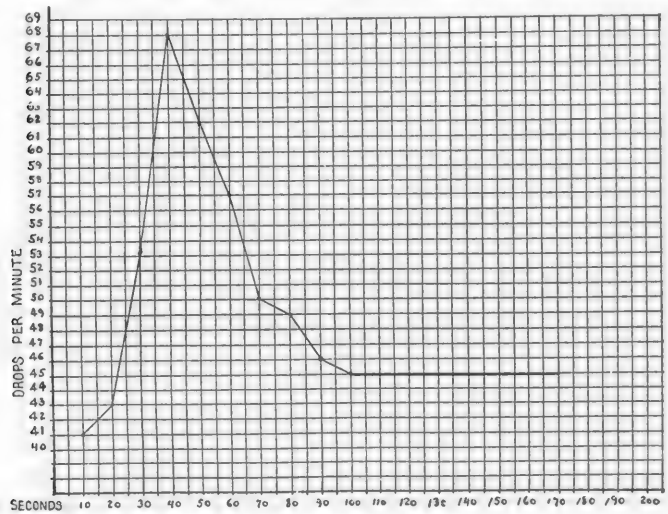
Sollmann, to whom apparently Brodie's work was unknown, described in the serum a vasodilator substance for the renal vessels acting on the isolated kidney. He also observed that the substance

was a coagulable proteid, but did not extend his investigation beyond the determination of this fact. He shows, however, that the increased flow from the vein is accompanied by a diminution in kidney volume, the vasodilator effect being therefore limited to the efferent vessels.

This probably accounts for Brodie's misinterpretation that there was a vasoconstriction of the kidney vessels and a vasodilatation of the peripheral vessels, when in reality there is a dilatation of both peripheral and renal vessels. The difference lies in the fact that in the case of the kidney it is the efferent vessels which are dilated, while in the limb the afferent vessels are more especially dilated.

NATURE OF THE DILATORY SUBSTANCE.

It is evident at the outset that the dilatation of the vessels of the perfused kidney must be regarded as the result of the activity of a substance other than that described by Brodie. His dilatory substance is potent only by reflex inhibition of the vasomotor center and is specific for the cat's vessels. The vasodilator substance described by us acts on the periphery and is not specific for the vessels of any one animal, and is found in the serum of all the laboratory

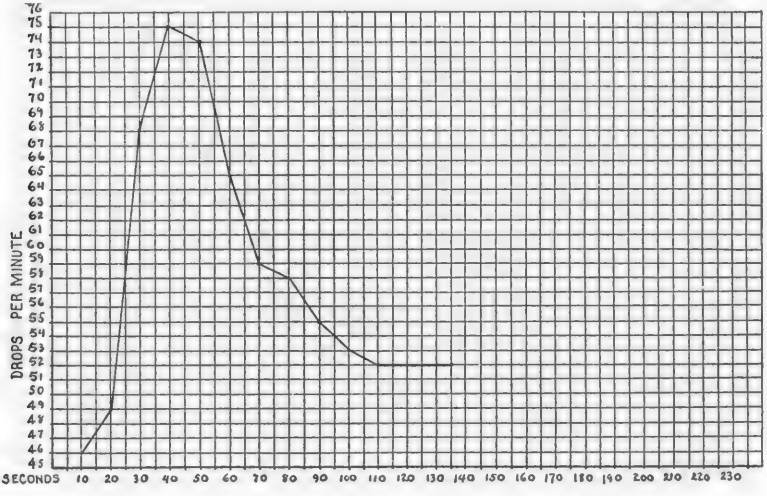


TEXT-FIG. 6. Effect of dog serum on dog kidney.

animals tested. We have found, further, that it is a constituent of the plasma and is not liberated during the process of clotting. This fact is brought out by the following experiments.

Two 20 c.c. samples of blood were taken from the same animal. To one was added 5 c.c. of a 0.2 per cent. solution of hirudin in Ringer's fluid. To the other was added 5 c.c. of Ringer's fluid alone. After the sample to which Ringer's solution alone was added had clotted, the serum was pipetted off and injected into the perfused kidney. It produced the typical dilatory effect (text-figure 6).

The sample of blood that was prevented from clotting by the addition of hirudin was immediately centrifugalized, the plasma filtered, and 2 c.c. were injected as before. An identical result was obtained; namely, a pronounced vasodilatation which equalled that of the serum alone (text-figure 7).



TEXT-FIG. 7. Effect of dog hirudin plasma on dog kidney.

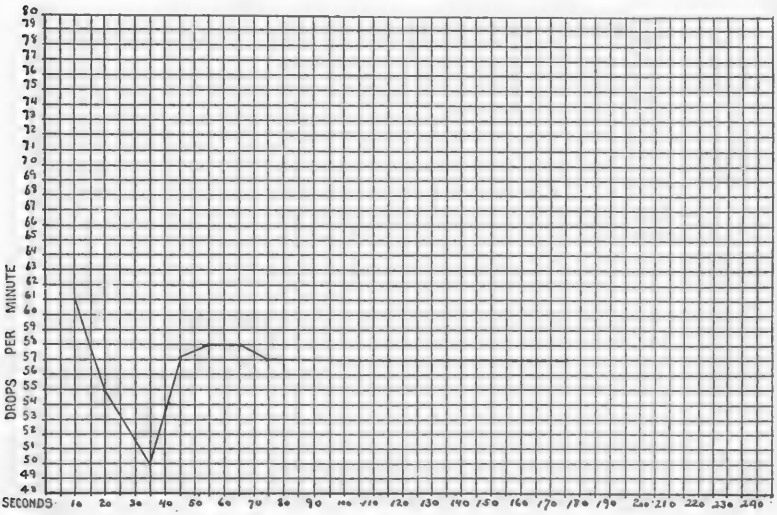
There remains the possibility that in the plasma sample the dilatory effect was produced by the hirudin used to prevent clotting. This point was readily settled by the injection of hirudin alone in the concentration used in the plasma. No change was produced in the rate of flow.

Having thus found that the vasodilator substance is a constituent of normal plasma, we next attempted to determine its origin and chemical properties.

Recent work on the internal secretion of the pituitary gland has

indicated that in this gland a substance is present that causes an increased flow of blood from the renal vessel in addition to an increase of the kidney volume as measured oncometrically (Magnus and Schäfer (9)). This substance is present only in the posterior lobe and can be extracted either by water or glycerin. It produces, in addition, a constrictor action on the vessels of the extremity as was shown by Oliver and Schäfer. They found that it diminishes the flow through a perfused frog.

Can the dilator effect of normal plasma on the kidney be attributed to the presence of this substance? It can be satisfactorily shown that the active substance obtained from the pituitary gland is not responsible for the dilatory effect on the renal vessels, for it has been found that pituitary extracts retain their activity after boiling, whereas we have observed that plasma or serum after coagu-



TEXT-FIG. 8. Effect of dog serum (boiled) on dog kidney.

lation by boiling loses its dilator property. The following experiments bear this out.

The serum was diluted three times with Ringer's solution, brought to the boiling point, and filtered through a Berkefeld filter. Two c.c. were injected and the result was compared with that obtained after the injection of a similar

amount of normal serum diluted to the same extent. The normal diluted serum caused a dilatation, but in text-figure 8 it will be seen that the boiled diluted serum produced, not a vasodilatation, but a vasoconstriction.

While these experiments serve to differentiate the two substances, they introduce a new factor that will be discussed later.

A recent work by Ogawa (10) dealing with the dilator effects of suprarenin makes it clear that suprarenin stimulates both the vasoconstrictor and the vasodilator nerve terminations, and the weaker the solutions, the more pronounced, relatively, is the dilator action. At a certain concentration no primary constrictor effect may be elicited, the only observable result being dilatation. He has proved that this holds good for the vessels of the intestine, and in some experiments he has found it to occur also in the kidney.

That a dilator action such as that described by Ogawa should account for our results seems *a priori* highly improbable, but the fact that suprarenin, like pituitary extract, resists boiling, indicates at once that we are not dealing in our experiments with suprarenin.

Since the vasodilator substance is precipitated by boiling, we infer that it belongs to the proteids. The filtrate, after precipitation by alcohol and dilution with Ringer's fluid, is inactive, indicating that the substance is present in the precipitate. We have not, however, been able to recover the active substance when once precipitated. It is not identical with the complement of the blood serum, since heating to 70° C. for four hours does not destroy it, although there is a noticeable reduction in its activity.

The vasodilatin described by Popielski (11), which he regards as the depressor substance of Witte's peptone, differs both chemically and physiologically from the dilator substance that we have found in plasma and serum. This vasodilatin has been carefully studied by Popielski and his pupil, and it has been shown by them to be identical with the depressor substances that can be extracted from the brain, intestines, and other organs. It is not a proteid and resists boiling in alkaline solutions. It does not act specifically on the kidney, but exercises its influence on the peripheral vessels as well.

As we have already pointed out, boiled serum loses its vasodilator property, but it is not inactive, for it can cause a well marked vasoconstriction (text-figure 8).

It might be contended that the change in the concentration of the inorganic elements of the serum incidental to boiling may have given rise to the constrictor effect that was evident after coagulation of the proteid. It is now well known, especially from the work of Hooker (12), that an increase in the sodium salts tends to cause dilatation of the blood-vessels, while an increase in the calcium salts produces constriction. Although boiling does not alter the relative concentration of the salts in blood serum, the possibility that an increase in the concentration of these salts might cause the constrictor effect was considered, and several injections of serum were made in which the original volume was restored by the addition of distilled water, but decreasing the concentration of the salts altered in no way the intensity of the reaction.

A more likely explanation is that there are two substances in serum, one of which, the dilator, is precipitated by boiling, while the constrictor remains in the filtrate. If this is the case, the two substances are so apportioned that the dilator is the more powerful. What one really measures after the injection of normal serum is the resultant of the activities of two substances that oppose each other.

If a vasoconstrictor substance is present in serum before boiling, it may be the normal suprarenin of the serum or it may be a substance liberated during clotting.

That the vasoconstriction is not produced entirely by suprarenin is proved by experiments with apocodeine to be described later. It seems highly probable, therefore, that a true vasoconstrictor substance is actually present in the serum and that its existence becomes manifest only after the removal of the vasodilator.

In this opinion we are strengthened by the study of the form of the plotted curve obtained by injecting unboiled normal serum. This curve (text-figure 3) has a sharp apex formed by the quick rise to a maximum and the sudden fall toward normal. At first the curve descends very rapidly but soon more gradually. We have evidence that the vasoconstriction (represented by the drop in the curve) begins before the serum has passed through the organ, for in many experiments in which the serum was blood-tinged due to the laking of a little blood in defibrinating it, this color of the serum has enabled us to recognize it in the fluid escaping from the vein. Our

interpretation of the curve is that the dilator substance, on account of its shorter latent period, is unrestrained in its action for the first few seconds after it reaches the vessels and thus causes the very sudden increase in the flow. Soon, however, the constrictor substance becomes active and rapidly diminishes the flow until an equilibrium is established, after which the outflow gradually drops to normal as the serum is washed out.

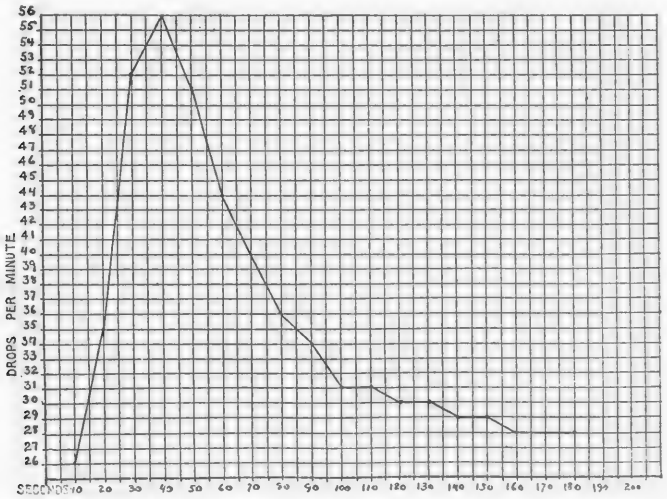
THE SEAT OF ACTION OF THE VASODILATOR AND THE VASOCONSTRICTOR SUBSTANCES.

The work of Dixon (13) has furnished a method whereby the action of a vasodilator or vasoconstrictor substance can be localized either on the sympathetic nerve terminations or on the muscle coat. Apocodeine hydrochlorid injected into the intact animal paralyzes the sympathetic endings in the arterial coat with the result that a subsequent injection of suprarenin is without effect. We therefore added to the stock Ringer's solution a sufficient amount of apocodeine to make a strength of 0.1 per cent., and perfused the kidney as before. In a few minutes the outflow from the vein was uninfluenced by the injection of even 1 to 10,000 adrenalin chlorid solution (Parke, Davis and Company). Very strong doses of the drug may, however, produce some slight constriction, but the marked difference in response of the vessels before and after the action of apocodeine is so pronounced that for concentrations of suprarenin approximating that of the blood serum it may be held that apocodeine completely abolishes its constrictor effect.

Effect of Serum after Apocodeine.—If two cubic centimeters of normal serum be injected into the vessels of a kidney perfused with Ringer's solution containing apocodeine, the effect is to produce the vasodilatation that is usual when the organ is perfused with Ringer's solution alone.

In an average of five experiments the increased outflow amounted to 120 per cent. In text-figure 9 the three phases previously described are to be distinguished. We have received the impression that paralysis of the sympathetic nerve terminations results in a greater percentage of increase in flow for the same amount of the serum injected. The experiments, however, are insufficient in num-

ber to justify a positive statement, since very great variations are observed normally both in regard to the sensitiveness of the kidney and to the potency of serum. *A priori* a greater percentage increase in the outflow after apocodeine is to be anticipated since the adrenalin content of the serum tends to lessen the degree of dilata-



TEXT-FIG. 9. Effect of rabbit serum and apocodeine on rabbit kidney.

tion produced. We are concerned, however, in the present work more with the qualitative than with the quantitative effect, and the results are so distinct as to warrant the conclusion that the dilator substance in normal serum acts directly on the muscle coat.

In a similar manner it is easy to determine that the vasoconstrictor substance has an analogous mode of action, for boiling destroys the vasodilator substance, while the vasoconstrictor remains active. The injection of boiled serum into the vessels of the kidney whose vasomotor endings are put out of function by apocodeine produces a well marked diminution in the flow from the organ, indicating that the vasoconstrictor substance also acts directly on the muscle coat.

THE RESPONSE OF PERIPHERAL VESSELS TO INJECTIONS OF BLOOD SERUM AND PLASMA.

The posterior extremities of the frog and rabbit were used to investigate this point—the technique for the rabbit's limb being essentially the same as that used for the kidney. The limb was severed from the body in such a way that the attachments of the muscle of the thigh were left intact, the object being to prevent oozing of the perfusion fluid from cut muscle surfaces. The limb was perfused through the femoral artery and the flow from the vein allowed to drop on the drop recorder. For the frog we used the method described by Trendelenburg.

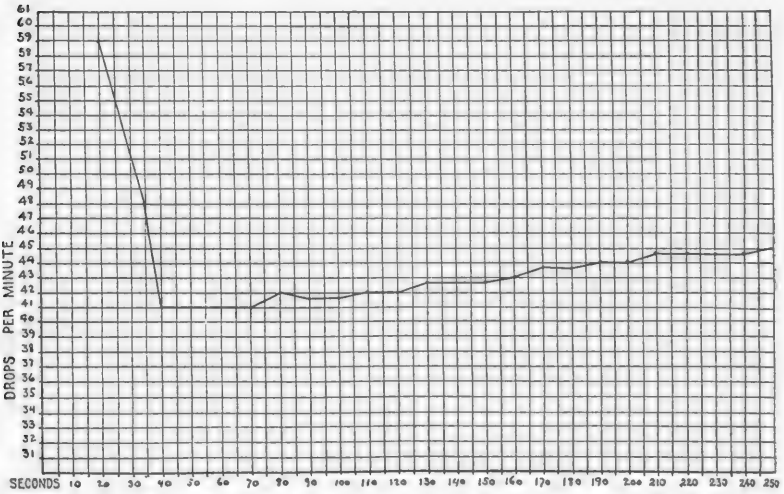
The injection of two cubic centimeters of rabbit serum into the vessels of the extremity produces an immediate constriction, the maximum of which was obtained in from one to three minutes. The interval between the time of injection and the attainment of the full constrictor effect varies with the amount of serum injected, and especially with the sensitiveness of the preparation. The smaller the dose, the sooner is the point of maximum constriction obtained.

Great variations are found in the constrictor effect of the same serum on different preparations. While the frog's vessels are generally more sensitive than those of the rabbit, it is not unusual to find two cubic centimeters of rabbit serum in one case producing a marked diminution of outflow amounting almost to complete stoppage, while the same serum in the other limb of the same animal may produce only a moderate slowing.

The plotted curve after the point of maximum intensity is reached, apart from its inversion, differs markedly from the corresponding curve of the dilator substance acting on the kidney. In the latter two phases are present, and in one the return to the normal flow is more rapid than in the other. The apex of the constrictor curve is never pointed as in the renal vasodilator. There is a gradual and regular return to normal following a period in which the flow has remained for a number of minutes at a constant low level. The effect of the constrictor is more lasting than that of the kidney dilator, and it may sometimes last as long as twenty minutes before the original flow is established.

The coronary vessels respond in a similar manner. O'Connor in

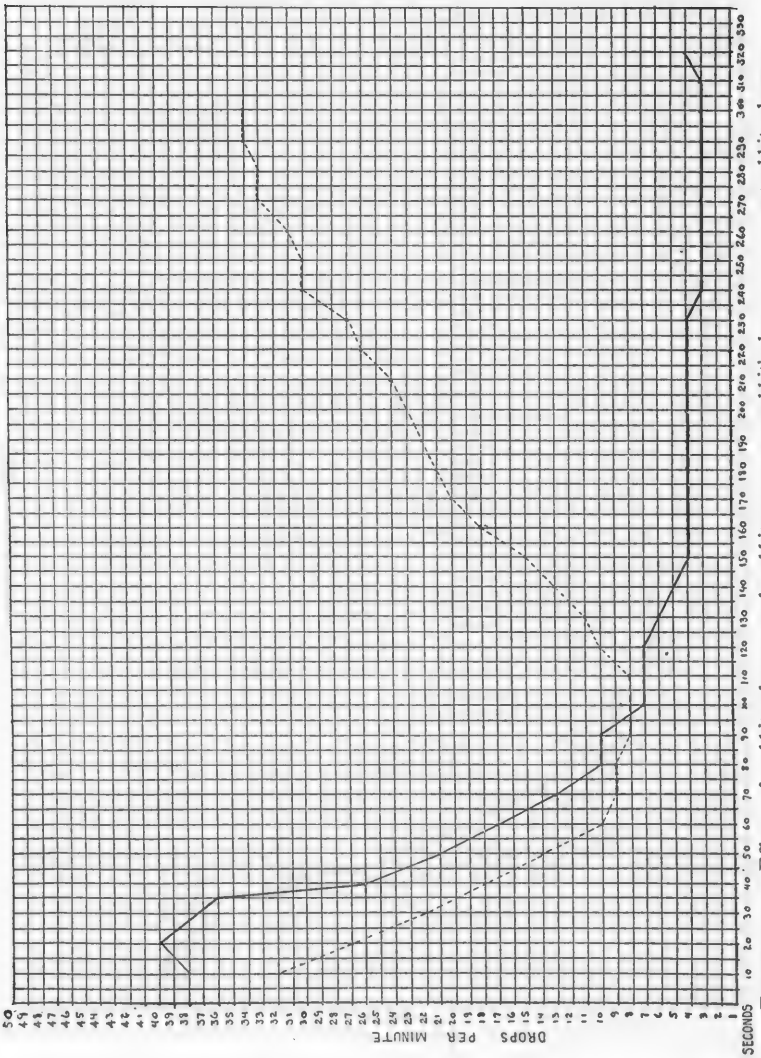
analyzing the vasoconstriction of peripheral vessels found that it equalled that produced by a concentration of 1 to 10 to 1 to 5 of pure suprarenin in the Trendelenburg frog preparation. The same serum when tested on the uterus preparation gave a suprarenin equivalent of 1 to 300,000 to 1 to 200,000. The conclusion drawn from the variability of these results was that other vasoconstrictor



TEXT-FIG. 10. Effect of rabbit serum on rabbit's leg.

substances were present in the serum which acted more powerfully on the uterus than on the Trendelenburg preparation. That this conclusion was correct he proved by destroying the suprarenin of serum by the method of Embden and von Furth. This method consists in the passage of a stream of oxygen for two to six hours through the serum at body temperature. Serum thus treated still retains the power of constricting the vessels of the frog's extremities. O'Connor further showed that this constrictor substance is found in serum and not in plasma, and that it is formed during the process of clotting.

We have repeated O'Connor's experiments on the relative activity of serum and plasma on the vessels of the rabbit's limb and on the frog, and our results confirm his. Thus in text-figure 11 are given



TEXT-FIG. 11. Effect of rabbit plasma and rabbit serum on rabbit's leg. ..... = rabbit plasma. ——— = rabbit serum.

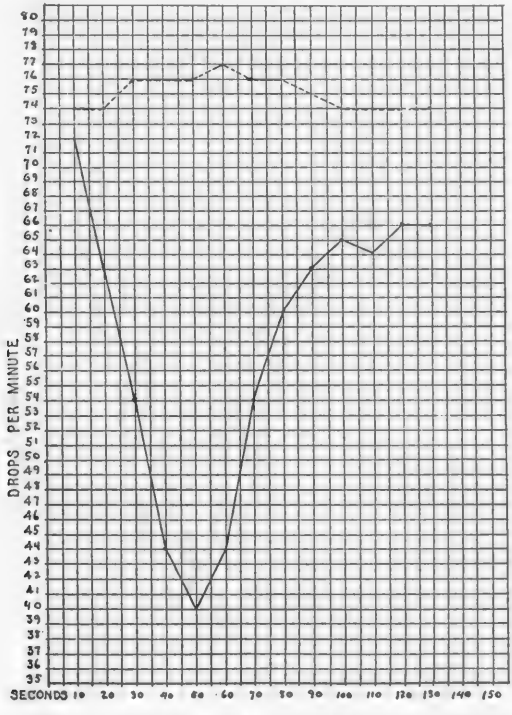
the effects of hirudin plasma and of serum on the same preparations.

It will be noticed that the former produces much less constriction than the latter. In none of our experiments have we observed any

indication of a vasodilator substance for peripheral vessels. The same serum which produces a vasodilatation of the renal vessels causes a constriction of the vessels of the limb.

By the use of apocodeine we have been able to differentiate more exactly between suprarenin and the vasoconstrictor substance liberated during clotting. After the vasomotor terminations have been paralyzed by apocodeine, injections of serum still produce a well defined reduction in the outflow from the veins of the extremities, proving, in the first place, the presence of a constrictor substance other than suprarenin; and, secondly, its direct action on the muscle coats.

A further proof of the presence of a vasoconstrictor substance produced by coagulation of the blood is furnished by the following



TEXT-FIG. 12. Effect of rabbit plasma and rabbit serum on rabbit's leg paralyzed by apocodeine. ..... = rabbit plasma. ——— = rabbit serum.

experiment. If suprarenin is the only active constrictor present in plasma, then the injection of plasma into the vessels of the limb perfused with Ringer's solution containing apocodeine should produce no change in the rate of flow, while serum should produce a diminution in the rate provided the substance given off during clotting acts by direct stimulation of the muscle coats of the arteries.

Text-figure 12 shows that such is indeed the case. Diminution in the flow was produced by serum, but not by plasma.

The constrictor substance for peripheral vessels is not influenced by boiling and is soluble in alcohol. It is therefore not a proteid of the albumin class, as is the dilator substance acting on the kidney vessels, but beyond this we have not attempted to define its chemical composition.

CONCLUSIONS.

1. In plasma there exists a vasodilator substance specific for the vessels of the kidney.
2. This substance is a proteid of the albumin class and is precipitated by boiling and by alcohol.
3. It is present also in the serum.
4. It acts directly on the muscle coats of the arteries.
5. The process of clotting of the blood liberates a constrictor substance that acts on the renal vessels and also on the vessels of the limb.
6. This constrictor substance is not a proteid; it resists boiling, is soluble in alcohol, and acts directly on the muscle coat.

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PLATE 8.

FIG. 1.

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EXPLANATION OF PLATE 8.

FIG. 1. Effect of dog serum on rabbit kidney.

EXPERIMENTAL BRONCHOPNEUMONIA BY INTRA-BRONCHIAL INSUFFLATION.*

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Plates 9 and 10.

It is now generally assumed that the pathogenic organisms present in the lesions of the various forms of pneumonia form only one link in the chain of conditions which give rise to the development of these diseases. The natural or acquired specific resistance of the individual and of the species, the state of vitality of the individual and of the organ under consideration, are factors which, it is believed, determine the outcome of a bacterial invasion. This view was strengthened by the frequent failures in the attempts to produce pneumonias experimentally by using pure cultures of the corresponding microörganisms; and, in the case of bronchopneumonia, this view certainly seems quite plausible, since in most instances this form of pneumonia is associated with some other pathological condition. Recently, however, in forty-two out of forty-four dogs, Lamar and Meltzer[1] succeeded in producing lobar pneumonia with pure cultures of the pneumococcus. The injection of the cultures was made by a new method, that of intrabronchial insufflation. The two failures were due to some faulty step in carrying out the new technique. In the experiments of Lamar and Meltzer the animals were neither selected nor prepared in any manner, and the microörganism was, apparently, the essential factor in the causation of lobar pneumonia. The favorable outcome of these experiments suggested the advisability of applying the same method to the action of organisms usually found in association with bronchopneumonia.

* Received for publication, April 22, 1912.

[1] Lamar, R. V., and Meltzer, S. J., *Jour. Exper. Med.*, 1912, xv, 133.

EXPERIMENTAL PART.

We have carried out a series of intrabronchial insufflations with the streptococcus and the influenza bacillus. In its essentials the method consists in introducing a tube through the mouth, larynx, and trachea, deeply into a bronchus, and then injecting the culture through the tube. The details of this method are recorded in the paper of Lamar and Meltzer.[2]

Experiments with the Streptococcus.—The virulence of several strains of streptococci isolated from cases of empyema and bronchopneumonia was tested in white mice, and the strain finally chosen was obtained at autopsy from the heart's blood and lungs of a fatal case of bronchopneumonia. When inoculated intraperitoneally in doses of 0.5 of a cubic centimeter of a twenty-four hour bouillon culture, this strain caused in twenty-four hours the death of a mouse weighing fifteen grams. Cultures of this organism were injected intrabronchially in twenty dogs in quantities varying from five to thirty cubic centimeters. In some experiments the cultures were enriched ("angereichert") by centrifugalizing them and resuspending the organisms. In this way the quantity of culture injected was made to contain two to six times the number of cocci present in the uncentrifugalized broth.

Of the twenty animals experimented with, one died within twenty-four hours. This was the only case in which the pneumonic lesion contained, besides the streptococcus, the bacillus of distemper. The other nineteen dogs were killed at intervals varying from twenty hours to six days. All had typical pneumonic lesions in various stages of progression and resolution depending upon the time elapsing between the injection and the killing of the animal. These lesions contained the streptococcus in pure culture.

The clinical course in these experiments was mild and of short duration and did not differ greatly from that described by Lamar and Meltzer for the non-fatal cases in the experimental infection with the pneumococcus. A few hours after the intrabronchial insufflation the temperature of the animal rose in some instances to above 40.4° C., but it came down, as a rule, on the following day and the animal soon appeared practically normal.

[2] Lamar, R. V., and Meltzer, S. J., *loc. cit.*

Lesions.—In the dogs killed from twenty to twenty-four hours after injection, the areas of consolidation were limited usually to the right posterior lobe, but in addition they were sometimes scattered in the subcardiac and upper lobes of the same lung, and were occasionally found in the other lung. The extent of the consolidation varied with the size of the dose injected; following a small dose the lesion involved sometimes only a small and superficial area, while after a large dose as much as half the lobe might be consolidated. The lungs were pink in color, contrasting with the dark red of the consolidated areas which were distinctly lobular in distribution, aerated lobules being discernible between the solid ones. The section was moist, smooth, and mottled, passing gradually by an irregularly ragged line into the aerated lung substance of the rest of the lobe. The pleura was not inflamed in any case, though its luster was impaired in several instances. In no case was there a central pneumonia. The solid areas and the lung adjacent to them were always deeply congested. Edema was slight. Smears from the solid areas showed many streptococci. Cultures from these areas gave a fairly profuse growth. From the uninvolved lung and from the heart's blood there was no growth.

After forty-eight hours the areas of consolidation were larger than after twenty-four hours, thus showing that the lesion was a progressive one. The dogs killed at the end of two days showed an extensive area of consolidation in the right posterior lobe, as much as two thirds or three fourths of it being solid, and usually there were consolidated areas in the right middle, left posterior, and left upper lobes also. The adjacent bronchial lymph nodes were but slightly swollen, were red in color, and were moist on section.

On the third day resolution was going on and the lungs showed consolidated areas of narrow and superficial extent only, the periphery shading gradually through a more or less congested area into the aerated lung substance.

On the fourth and fifth days resolution had progressed so that only a dark discoloration was left to outline the areas originally consolidated. In only one case was a narrow strip of quite solid pneumonia found on the sixth day, and to this animal the very large dose of thirty cubic centimeters had been given.

The right lung was involved in nineteen of the twenty cases. The left lung was also involved in eight instances, and in one case only was the pneumonia limited to the left posterior lobe. In twelve cases the areas of consolidation were scattered in two or more lobes of the lungs, and in a general way it may be said that the larger the quantity of culture fluid injected, the more scattered and the more extensive were the lesions; and the more numerous the organisms, the more severe the lesion. The enriched ("angereichert") cultures produced a more diffuse and intense inflammation, but did not delay resolution, for on the fifth day one case which had received a culture enriched six times was completely resolved. Streptococci were abundant in the smears from the consolidated areas on the first and second days and grew well in cultures. On the third day they were no longer profuse, and no growth was obtained on the fourth day. In no instance did the heart's blood give a growth of streptococci.

Microscopic Examination.—On microscopic examination within twenty-four hours the picture varied according to the size of the dose injected. In the least severe case the animal received only five cubic centimeters of culture. In this instance the exudate consisted chiefly of large mononuclear cells (the desquamated epithelial cells of the alveolar walls), with comparatively few polymorphonuclear leucocytes, and very little fibrin. The capillaries in the alveolar walls were intensely congested, but no red blood cells were present in the alveolar contents. The cells lining the bronchial walls were intact and no inflammatory infiltration was noted in these walls. The most densely packed solid alveoli were grouped around the smallest bronchioles, and in the areas which were the most solid, polymorphonuclear leucocytes were relatively more numerous than in the less solid areas, which, at the periphery of the lesion, shaded into lobules whose alveoli contained only granular coagulated serum and a few epithelial cells. Cocci were present in the alveoli in large numbers, and also in the bronchi,—many of which contained masses of exudate, evidently sputum.

A larger dose, fifteen cubic centimeters, had called forth a far more solid lesion in which the leucocytes were decidedly more numerous, and in which the walls of the smaller bronchi were distinctly

infiltrated. A still larger enriched dose, thirty cubic centimeters, had produced a very marked purulent infiltration, not only of the walls of the alveoli and bronchioles, but of the connective tissue septa, and even of the adventitia of the blood-vessels. There was very little fibrin present. The cocci were numerous.

On the *second* day the relative severity of the lesion had increased greatly, both in quantity and in quality. Thus the leucocytic infiltration had extended so that all the structures in a solid lobule were uniformly involved,—the alveoli, bronchi, blood-vessels, and septa—and the section gave a general impression of pus. This infiltration was most marked in the dog to which the largest dose had been administered. The desquamated epithelial cells were present in the alveolar exudate, but the leucocytes outnumbered them. Only a very small amount of fibrin could be detected. Beyond the solid areas some alveoli contained red blood cells, and all the capillaries were congested, as well as the smaller bronchial vessels. Cocci were still numerous, but phagocytosis was not marked.

On the *third* day resolution was progressing so rapidly that an area of bronchopneumonia caused by a small dose (five cubic centimeters) showed scarcely any packed lobules. There were very numerous examples of macrophage activity and a granular mass was present between the cellular remnants. In the more severe cases distinctly solid alveoli remained about a bronchiole, while the periphery of the lobules was becoming emptied of their exudate by phagocytosis and cell degeneration. Cocci were still present at this time.

On the *fourth* day only a few solid alveoli remained close around the bronchioles, and on the *fifth* day none were seen. Only a granular mass with a few leucocytes and epithelial cells was left in the alveoli, but the septa were still edematous and infiltrated with leucocytes, as were the alveolar walls. On the *sixth* day only one case was examined. This animal had received a large dose (thirty cubic centimeters) and in its lungs some well preserved exudate was seen in the alveoli around a bronchus, while almost all of the area that had previously been solid had undergone resolution.

There was no evidence of organization in any of these cases.

It is well to bring out here the difference between the pneu-

monia produced in the experiments of Lamar and Meltzer with the pneumococcus, and the pneumonia produced in the present series of experiments with the streptococcus. (1) In the pneumococcus infections there was a mortality of at least 16 per cent.; in our experiments there was practically no mortality. (2) In the pneumococcus infections there was a pneumococcus septicemia in all the fatal cases; no bacteriemia occurred in our streptococcus cases. (3) In the pneumonia lesions from the pneumococcus, the consolidated part of the lung was always air-free and lobar in character, no matter how small or large the affected area was; in the pneumonia lesions produced by the streptococcus infection, no matter how large the area, or how intense the inflammatory process was, aerated lobules were always discernible between the solid one; *i. e.*, the streptococcus pneumonia was "lobular." (4) In the pneumococcus pneumonia there was always a definite pleurisy present even in all the non-fatal cases, while it was practically completely absent in the twenty cases of the streptococcus infection. (5) In the pneumococcus infection the cut surface of the consolidated areas was rather dry and granular; in the streptococcus infection it was very moist and smooth. (6) Fibrin was an important element in the exudate of the lesions brought about by the pneumococcus infection, while it played practically no part in the pneumonia consolidation in our experiments. (7) In the pneumococcus pneumonia the walls of the bronchioles, although filled with exudate, were free from infiltration with leucocytes; while in the streptococcus pneumonia the bronchioles were intensely infiltrated by the leucocytes, especially in the more severe lesions. (8) Resolution progressed much more rapidly in the streptococcus pneumonia than in the pneumonic lesions produced by the pneumococcus.

The most essential points are the differences in the nature of the consolidation and in the fibrin formation.

ILLUSTRATIVE PROTOCOLS.

I. *Dog 2.*—Fox terrier, male; weight, 4,350 gm.

April 26, 1911, 12:30 P. M. Under ether anesthesia, a 5 c.c. dose of a twenty hour plain broth streptococcus culture was injected intrabronchially. 5 P. M. Temperature, 39.9° C.

April 27, 10 A. M. Temperature, 39.4° C.

April 28, 11:30 A. M. Temperature, 39.2° C. The animal at this time did not appear sick, but was killed with chloroform.

Autopsy.—The upper lobe of the left lung was normal. The posterior lobe was congested. The right posterior lobe was larger than any other, was swollen, and in its posterior third contained solid areas which involved the base also. There were a few very small and superficial solid areas in the right middle lobe. The pleura was quite normal. The bronchial lymph nodes on the left side were normal. On the right side they were a little enlarged, red in color, and very moist on section. On section the consolidated lung was smooth, patchy, red, and very moist. A thin red fluid exuded readily from the cut surface. The trachea was normal; the mucosa of the bronchi was reddened, and contained frothy, blood-stained fluid. Smears from the solid area showed numerous streptococci. In cultures from the fluid area a growth of streptococci developed, but there was no growth in the cultures from the heart's blood and from the left upper lobe.

Sections from the consolidated area showed that the alveolar exudate consisted of some large mononuclear (epithelial) cells and of a larger number of polymorphonuclear leucocytes, many nuclei being fragmented. The alveolar walls were infiltrated with these cells, and so were the walls of the small bronchi, the lumen being plugged with a mass of exudate. No fibrin was present.

II. *Dog 16.*—Yellow male; weight 4,800 gm.

June 6, 1911, 3 P. M. Under ether anesthesia, 30 c.c. of a twenty-two hour plain broth culture of streptococci, enriched to double strength, were injected into a bronchus. 6 P. M. Temperature, 40.4° C.

June 7, 9:30 A. M. Temperature, 39.7° C. 11:30 A. M. Animal killed with chloroform.

Autopsy.—The right posterior lobe was half solid and dark red, contrasting with the pink, aerated anterior portion of the lobe. The right apex and the subcardiac lobe also contained a solid area, and the upper half of the left posterior lobe was solid. The pleura was quite normal. On section the consolidated lung was mottled dark and light red, the light red areas being the smaller; they were aerated, while the dark ones were airless. Congestion and edema were very marked. Frothy fluid exuded from the bronchi. The bronchial nodes on both sides were congested and swollen.

Smears from the consolidated areas showed many streptococci. Cultures from these portions grew well; the heart's blood was sterile. Sections from the consolidated portion gave a general impression of pus; that is, the alveoli were filled with polymorphonuclear leucocytes and some epithelial cells. The leucocytes were numerous in the walls of the alveoli and in the bronchial walls; the smallest bronchioles showed degeneration of their lining cells, and the alveoli nearest to them were very densely packed with exudate in which there were a few short fibrin threads. All the vessels were distended with blood, but there were very few red blood cells in the alveoli.

EXPERIMENTS WITH THE INFLUENZA BACILLUS.

The relative frequency with which the influenza bacillus is found in cases of pneumonia led us to choose this organism for the production of experimental bronchopneumonia in dogs. The influenza bacillus may be present in pure culture in a human pneumonic lung, in which case the lesion is always of the lobular type. It may also be associated with the pneumococcus in lobar pneumonia, but it is found most frequently together with the streptococcus, the staphylococcus, or the pneumococcus in various combinations in cases of lobular pneumonia of the peribronchial type. It seemed a matter of some interest, therefore, to know how the intrabronchial injections of influenza bacilli would affect the lungs of the animal experimented upon.

A strain of the bacillus isolated from a case of leptomeningitis and virulent for rabbits and monkeys was used. It was grown on rabbit blood agar in Blake bottles and washed off in salt solution. The growth of one to three bottles, suspended in five to thirty cubic centimeters of salt solution, was injected. Eleven dogs were experimented upon and none died. It must be remembered that the dog has been found to be very resistant to influenza bacillus infection.

Within five or six hours after the injection of the culture, the dog's temperature rose to about 39.8° C.; in two cases it reached 40.2° and 40.7° C. On the following morning it had dropped to 39° or thereabouts, and then to 38.2° or 36.5° on the third day. In one case it remained about 39° C. for three days. Cough was not noticed and the animals were not apparently ill. They were killed at intervals that varied between twenty-one hours and six days after the injection.

The earliest lesion studied was twenty-one hours old. In this instance a small dose had caused scattered areas of consolidation in all the lobes of both lungs, but only one area was larger than 0.25 of a centimeter in diameter The largest area was in the right middle lobe and was three centimeters in length. The consolidated part was not deep, but was very solid, though distinctly "lobular" in character. All the solid areas were deep red in color, and there were many hemorrhages in the aerated lung beyond. The pleura was normal.

A more concentrated dose produced in the same time a far more striking lesion. In this case the entire right posterior lobe was solid and small consolidated areas were scattered in the subcardiac and upper lobes as well as in all the lobes of the left lung. These areas were distinctly solid, were dark red in color, hemorrhagic, and moist, but small areas of aerated lobules were still visible between the solid areas. Smears showed many bacilli which grew in cultures. The heart's blood gave no growth.

Forty-eight hours after the injection of the organisms the lesion was of the same character, solid and dark red, not sharply demarcated from the aerated lung around it. Hemorrhagic areas were present in the lung.

In three days resolution was going on, the solid areas were resilient, were smaller in extent, and not so firm. On the fourth day the condition was about the same, while on the sixth day the pneumonia had almost entirely disappeared. At this time, after a concentrated dose, some resolved areas and a solid mass occupying half of the right posterior lobe were seen.

Microscopic Examination.—At the end of the first day the lesion showed that the exudate consisted of epithelial cells peeled off from the alveolar walls, some red blood cells, and many polymorphonuclear leucocytes. The alveolar walls were clearly outlined by the distended capillaries, and were not infiltrated. The bronchial walls were quite normal, and the lumen contained exudate composed of red and white blood cells, granular debris, and many bacilli. Only a very small amount of fibrin was present in the alveolar exudate. In the less solid portions of the lesion, the alveoli contained much granular serum, a few red cells, and some epithelial cells. In the same period of time (twenty-four hours) a more concentrated dose caused a more marked leucocytic exudation. Many of the leucocytes with fragmented nuclei infiltrated the bronchial and alveolar walls. There was very little fibrin; many bacilli were present, and hemorrhages into the alveoli near the solid areas were very numerous.

At the end of the second day the exudate was even more markedly leucocytic in character, and a purulent infiltration was present in all the bronchial walls, interlobular septa, and the alveolar walls. All

the bronchi contained plugs of exudate and around the bronchi were densely solid alveoli. On the third day resolution had begun. The congestion had subsided, the phagocytic cells were numerous in the exudate, and all the leucocytes were undergoing fragmentation. Bacilli were still very numerous.

On the sixth day the lesion produced by a small dose was almost entirely resolved, some granular serum and cell debris remaining in some of the alveoli, while the purulent infiltration had subsided. A more concentrated dose had produced a lesion which, on the sixth day, showed resolving pneumonia, as well as large peribronchial areas of consolidation in which resolution had not even begun.

There was no pleural exudate in any one of these eleven cases. Cultures of influenza bacilli were obtained from the pneumonic areas on the first, second, and third days, but from the heart's blood no growth was obtained at any time.

In the influenzal bronchopneumonia, as in the streptococcal variety, the size of the dose of bacteria injected had a distinct influence on the pneumonic areas that developed. The most extensive and most severe lesions were produced by a larger and enriched dose, and in such a case resolution was also delayed as compared with that of a lesion caused by an injection containing fewer organisms. Hemorrhage and purulent infiltration are characteristic features of this variety of lobular pneumonia.

ILLUSTRATIVE PROTOCOLS.

I. *Dog 27.*—Black and white female; weight 3,700 gm.

June 6, 1911, 3 P. M. Under ether anesthesia, 10 c.c. of a suspension of influenza bacilli which had been grown in two bottles for twenty-four hours were injected intrabronchially. 6 P. M. Temperature, 40.2° C.

June 7, 9:30 A. M. Temperature, 39.6° C. 11.30 A. M. The dog was killed with chloroform.

Autopsy.—The posterior lobe of the right lung was swollen, large, solid, firm, and dark red. The entire lobe was involved. On section, this lobe was hemorrhagic, solid to the touch and moist, and revealed very few aerated lobules among the solid areas. The greater part of the lesion, however, was hemorrhagic rather than inflammatory. The pleura was normal. On the right upper lobe were scattered areas of consolidation. These were also present in the subcardiac and middle lobes. In the left lung there were solid areas in all the lobes. These solid areas were larger near the upper angle of the posterior lobe, and all of them were peribronchial and interspersed among the aerated lobules. The bronchial nodes were deeply congested and very moist.

Smears from the right posterior lobe showed many leucocytes and many Gram negative bacilli. The influenza bacillus grew in pure culture.

Microscopical Examination.—The alveoli were filled with peeled off epithelial cells, polymorphonuclear leucocytes, and some red blood cells; there was some granular fibrin in the exudate, but it was nowhere excessive. The smallest bronchi had lost their epithelial lining and there was leucocytic infiltration of their walls. The larger tubes, however, had normal walls, but contained aspirated masses of leucocytes and red blood cells. Empty alveoli were present next to solid lobules. Bacilli were very numerous in the alveoli and bronchi. Many alveoli at the periphery of the solid areas were filled with red blood cells, and many other alveoli contained serum only.

II. *Dog 29.*—Black male; weight 7,400 gm.

June 13, 1911, 11 A. M. Under ether anesthesia a 10 c.c. suspension of the twenty-four hour growth from three bottles of influenza bacilli was injected intrabronchially. 5 P. M. Temperature, 40.2° C.

June 14, 9:30 A. M. Temperature, 39.6° C.

June 15, 9:30 A. M. Temperature, 38.8° C.

June 16, 9:30 A. M. Temperature, 38.7° C.

June 17, 9:30 A. M. Temperature, 38.5° C.

June 19, 9:30 A. M. Temperature, 38.9° C. The animal was killed with chloroform.

Autopsy.—The only consolidation found was in the right lower lobe. This was about half solid, and beyond was a dark area which was evidently almost resolved. The consolidated portion was firm and smooth, and on section was very moist and of a greyish red color. The dark area was aerated and more congested than the rest of the lobe. All the remainder of both lungs was quite normal. The lymph nodes on the left side were normal. On the right side they were slightly swollen.

Smears and cultures were negative for influenza bacilli. Sections from the consolidated area showed that the alveoli were filled with epithelial cells and polymorphonuclear leucocytes. No fibrin was present. The capillaries in the walls were not distended, but leucocytes were present between the meshwork. All the bronchi, large and small, showed extensive degeneration of their lining cells and a leucocytic infiltration of their walls, this infiltration being continuous in places with the exudate in the surrounding alveoli. At the periphery of the consolidated area there was marked phagocytosis of the cell fragments by large mononuclear cells. In some cases whole red blood cells or leucocytes were contained in these macrophages. As the cells diminished in number a granular material occupied the alveoli, and there was a gradual transition from these areas to those of normal empty air cells. No bacilli were found in these sections.

The differences between the lesions produced by the injection of the pneumococcus into the bronchi and those caused by the influenza bacilli were as marked as were the differences between the lesions produced by the pneumococcus and those caused by the streptococcus. The fibrin was slightly larger in amount in the influenzal

pneumonia than in the streptococcal variety, but was by no means as marked a feature of the exudate as in the pneumococcus pneumonia. Hemorrhage into the alveoli was more marked in the influenza cases than in either the streptococcus or the pneumococcus lesions, and leucocytic infiltration was as severe. In gross appearance influenzal bronchopneumonia was distinctly patchy and lobular, not lobar in type, although the tendency was for a large portion of a lobe to be involved.

SUMMARY.

When intrabronchial insufflation of pure cultures of the streptococcus or of the influenza bacillus is properly carried out, it produces without fail a pneumonic lesion. This lesion is similar in its nature to the one known in human pathology as bronchopneumonia, and differs materially from the pneumonic lesion produced experimentally by the intrabronchial insufflation of pure cultures of the pneumococcus.

Considering the fact that none of the dogs used in the experiments with the pneumococcus and none of those used in the present investigation were selected or prepared in any way, the conclusion seems to be unavoidable that the proper invasion of the microörganism is the determining factor in the development of pneumonia, the condition of the animal being only a minor element in this regard.

Furthermore, since different organisms introduced in the same way and under conditions which are apparently the same produced distinctly different pneumonic lesions in animals of the same species, the further conclusion presents itself that the different types of pneumonia are produced by specifically different bacteria.

However, further investigation may show that the differences in the nature of the lesion are due rather to the degree of virulence of the causative microörganism than to differences in the species; that is, that different lesions may possibly be produced by organisms of the same species, provided they possess different degrees of virulence. Further experimentation may also show that the condition of the animal and of the affected organ which, in the onset and development of the pneumonic disease, is, perhaps, unimportant, may be the leading factor in determining the course and outcome of the disease.

EXPLANATION OF PLATES.

Plate 9.

Fig. 1. Streptococcal bronchopneumonia of seventy-four hours' duration; dose 10 c.c.; twenty-four hour bouillon culture.

Fig. 2. Streptococcal bronchopneumonia of forty-eight hours' duration; dose 10 c.c.; enriched three times.

Plate 10.

Fig. 3. Influenzal bronchopneumonia of twenty-four hours' duration; dose 10 c.c.; enriched twice.

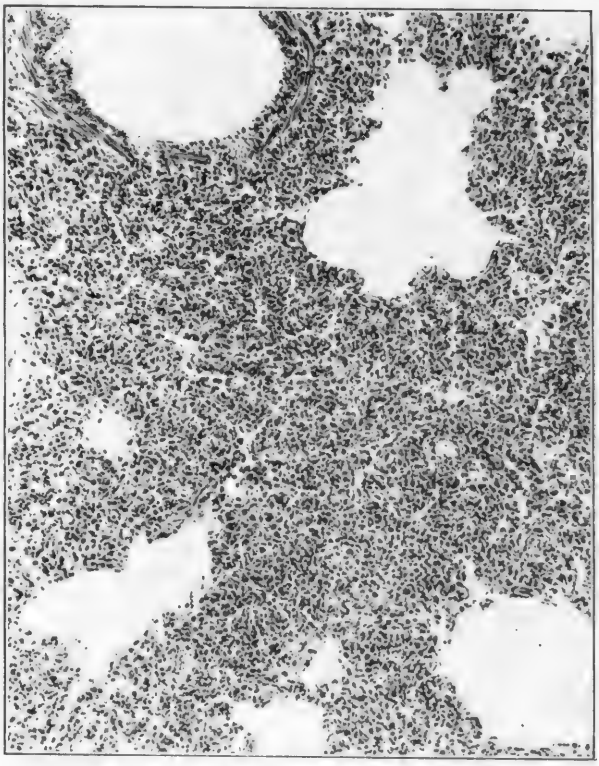

FIG. 1.

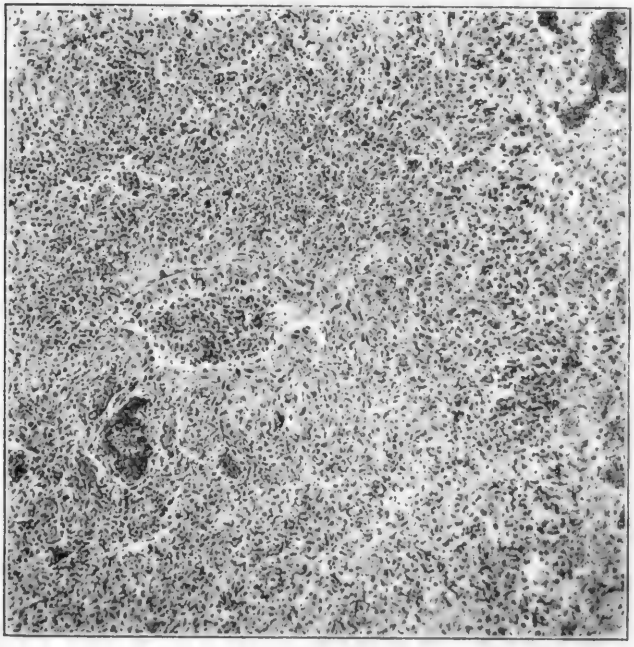

FIG. 2.

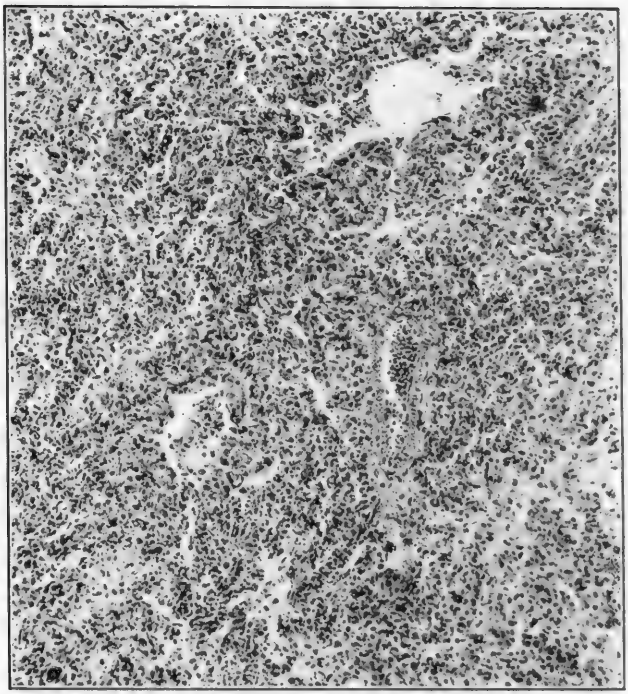

FIG. 3.

SOME OBSERVATIONS OF THE THORACIC DUCT LYMPH AFTER INJECTION OF OIL OF TURPENTINE INTO THE PERITONEAL CAVITY OF THE DOG.*

By ROBERT L. DIXON, M.D.

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The physical relation existing between serous cavities and the vessels of the lymphatic circulation has been studied in the past by many investigators, and efforts have been made to trace the paths taken by foreign material, soluble and insoluble, in leaving the abdominal cavity.

In this study we desired, particularly, to know if leucocytes that have entered the serous cavity as the result of an inflammatory process are returned to the circulation, and, if so, by what route. Some notice was also taken of the disposition made of insoluble pigment granules introduced into the abdominal cavity. In these experiments healthy dogs were used.

In order to establish the process of inflammation in the serous cavity, five to ten cubic centimeters of oil of turpentine were injected under morphin anesthesia through the abdominal wall by means of an ordinary syringe. In several instances a red dye was conveyed along with the turpentine into the abdominal cavity. At different intervals after the injection, the animals were examined.

Under anesthesia (chloretone) the thoracic duct was isolated in the neck, and a cannula introduced. Total and differential counts of the cells in the thoracic duct lymph were made in the usual way. The abdominal cavity was opened under anesthesia, care being taken to prevent blood from mixing with the fluid collected in the peritoneal cavity. Counts and study of types of cell in the effusion were then made. The observations and conclusions based upon this set of experiments are briefly stated in this paper.

* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, April 15, 1912.

The amount and nature of the fluid found varied greatly with different intervals. While the changes as determined in different dogs were not identical, yet there existed a general relationship between the factors involved.

The *first or earliest effect* noted was a marked congestion of the vessels of the serous coat, this being accompanied by an effusion. The effusion had a very pale yellowish color, was of a watery consistency, and contained very few cells. These cells were of the small mononuclear type. After four to six hours the fluid clotted in a test-tube, forming a very soft, jelly-like mass.

It was not possible to determine the absolute number of cells per cubic millimeter of this fluid because of the fact that as the animals lay for some time in a given position the cells settled, and no particular quantity truly represented the total. Nevertheless, from the counts made of the cells in this early fluid the number was probably not more than two to three hundred per cubic millimeter.

Four to six hours after the injection of the turpentine, the cover-glass smears showed very few cells. Practically without exception these were the small lymphocytes and showed no marked variation in size. Red blood cells were absent. The fluid present was about fifty cubic centimeters.

At the end of the *second day* the effusion had increased to two hundred cubic centimeters. The color was more nearly pink, due to the increased number of red blood cells. The density of the fluid was greater, and it clotted in one to two hours into a more compact mass. The calculated number of the white cells per cubic millimeter was about ten thousand.

These cells varied greatly in many respects. The majority were mononuclears. Most of these were small lymphocytes and some were very small indeed, but many were unusually large, being much larger than the cells ordinarily found in the lymph or blood. Of these very large mononuclear cells there were about a dozen to a cover-glass. In diameter these cells were approximately thirty to forty microns. They had a large, compact, blue-staining nucleus with a peripheral band of protoplasm stained bright pink. These were probably mesothelial cells.

All the cells showed the effects of soaking in the fluid. This was

indicated especially by their frayed and indefinite outlines. Many of the cells were phagocytes and contained red blood cells, pigment, and cell detritus. Red blood cells were very numerous.

At the end of the *third day* the effusion amounted to two to four hundred cubic centimeters. The density and the cellular content were greater than on the second day and within half an hour the fluid clotted into a rather firm mass. It was of a light creamy color and so stringy as to make it difficult to make thin cover-glass smears. The smears showed a relative increase in the number of polymorphonuclear cells. There were many mononuclear elements showing much disintegration, and a great amount of a stringy fibrinous substance. The number of red cells was much decreased.

On the *fourth day* the amount of the fluid was much less, of a thicker, more creamy consistency, and clotted into a firm mass within half an hour. There was now a definite preponderance of polymorphonuclear cells. There were also, however, many large mononuclear cells, usually with loosely arranged, palely stained nuclei. Red cells were practically absent.

On the *fifth day* the fluid had practically disappeared, and all that remained was a layer of fibrin. This was on the peritoneum, but especially on the diaphragm and on the surface of the liver and spleen. In the meshes of this fibrin were many pigment granules either free or enclosed within phagocytes.

Evidently it is not possible to compare the output from the thoracic duct in these instances with what it was before the injections were made. But from a knowledge of the findings in a great many cases it is possible to assume that certain types of cells were present in certain relative proportions, and to consider wide variations from this standard as unusual and perhaps as a consequence of certain factors and agencies employed in the instance in question.

The most extensive and detailed enumeration of the cell content of the lymph from the thoracic duct has been reported from this laboratory by Rous.[1] His report is based upon a careful consideration of the number and types of cells found in the lymph from twenty-three dogs in which, as far as could be ascertained, no complicating conditions existed.

[1] Rous, Peyton, *Jour. Exper. Med.*, 1908, x, 537.

My counts of the cells of the lymph can well be compared with the standard established by the findings of Rous. The observations of Delamere, and of Biedl and Decastello, as well as my own in a previous set of experiments, are also taken into account in this connection. Rous[2] states: "Mast cells are not a constituent of the lymph of the normal dog. Polymorphonuclear neutrophiles are only present as a result of blood admixture. Lymphocytes, by which are meant non-granular cells with a round or oval nucleus smaller than an entire polymorphonuclear neutrophile from the same animal, form an average of 87.6 per cent. of the cells." The percentage goes as low as 69.8 per cent. and as high as 96.8 per cent. "Typical transitional forms are rare in the lymph. . . . Large mononuclear cells—non-granular mononuclear elements larger than the lymphocyte as above defined—average 5.2 per cent. of the lymph's leucocytes." From the differences in the cell counts in individual dogs it is evident that in normal animals great variations from these average percentages occur. Since the variations are normally so great, the proportions of the cells must be altered extremely before we can be sure that a given agency has produced a noteworthy effect.

I have made a total and differential count of the cells in the lymph of the thoracic duct in each of my cases, the results of which are given in table I. By comparing the data in this table with those already cited, we may draw our conclusions.

If such a table could be made to apply to a single animal over a period of five days, and the test repeated on as many animals as are here represented, it would be very easy to form rather definite conclusions as to the types of the cells and the degree of variation in the cell formula of the lymph under varying conditions and agencies. Such procedures, however, do not seem to be practicable.

We can do little more than compare the relative proportions of the various elements in different animals and the approximate rates of flow.

The *small mononuclear* cells, which primarily constitute the bulk of cells in the lymph, here show a range of from 63.8 per cent. to 92.4 per cent. These extremes cannot be said to be far from the

[2] Rous, Peyton, *loc. cit.*

TABLE I.

Total and Differential Counts of Cells in the Thoracic Duct at Varying Intervals after Injection of Oil of Turpentine into the Peritoneal Cavity of Dogs. Each Set of Counts Represents a Different Animal. Eleven Animals Were Used.

After injection.	Small mononuclears.		Large mononuclears.		Polymorphonuclear neutrophils.		Eosinophils.		Transitionals.		Total counted.	Total per c.mm.	Flow in 10 min. in c.c.
	No. of cells.	Per cent.	No. of cells.	Per cent.	No. of cells.	Per cent.	No. of cells.	Per cent.	No. of cells.	Per cent.			
4 hrs.	476	84.5	59	10.4	7	1.2	9	1.6	12	2.1	563	8,650	7.0
6 hrs.	403	79.6	77	15.2	6	1.1	3	0.56	16	3.1	505	5,500	12.5
36 hrs.	417	73.5	105	18.5	12	2.1	7	1.2	26	4.5	567	9,975	9.5
2d day	348	71.6	91	18.7	27	5.5	2	0.4	18	3.7	486	7,500	14.0
2d day	240	63.8	73	19.4	19	5.0	12	3.2	32	8.5	376	10,400	7.0
3d day	427	73.7	96	16.6	7	1.2	6	1.0	42	7.2	578	9,225	13.0
3d day	318	70.4	85	18.8	21	4.6	4	0.9	23	5.0	451	12,600	13.5
4th day	409	83.4	28	5.7	26	5.3	18	3.7	9	1.8	490	11,900	8.0
4th day	381	89.4	19	4.4	14	3.3	3	0.7	9	2.1	426	19,100	11.0
5th day	412	91.9	15	3.3	5	1.1	11	2.4	5	1.1	448	6,475	7.5
5th day	341	92.4	13	3.5	9	2.4	2	0.54	4	1.09	396	11,500	5.0

percentages taken as a standard, although the lowest count is below the minimum percentage obtained by Rous.

The variations of percentage with reference to the effusion are significant. At first when the amount of effusion was small and the cells contained in it were few, the percentage of mononuclear cells in the lymph was relatively high. Later, as the *effusion* became characterized by mononuclear cells of this type, the percentage in the *lymph* diminished. This is, perhaps, not what we should expect if we were to take the view that the cells of the effusion are supplied from the blood, and that the supply in the blood is maintained by the cells coming from the thoracic duct. But even from this point of view it seems possible to explain the findings.

The first effect of the injection of the turpentine is a demand upon the protective forces of the body. This is evidenced by the prompt pouring out of a fluid that acts as a diluent and perhaps as an antagonist chemically to the irritant agent. Simultaneously with this there is an increased activity on the part of the tissues or organs which furnish the cellular elements. Hence a high percentage of the elements in the lymph at this time consists of cells that are about to be passed into the blood and later into the peritoneal cavity. But

there is no doubt that many of the cells go to the seat of activity without passing through the ordinary channels. This is especially true later in the process, and accounts for the continued relative increase in mononuclear cells in the effusion after the relative decrease in the thoracic duct. Later in the process the percentage of the small mononuclear cells in the lymph rises again to a high mark, due perhaps to the reverting of the supply into the usual channel and to the taking up of many of these cells from the peritoneal cavity. That the mononuclear cells are taken up from the peritoneal cavity is evidenced by the great numbers of poorly stained, loosely formed, and misshaped cells of this type that are seen in the smears from the lymph at this time.

The *large mononuclears* maintain a ratio that is practically the reciprocal of that of the small mononuclears. The percentage of the large mononuclear cells in the lymph is high immediately before and during the time when their number in the peritoneal fluid is greatest. Late in the process, however, the ratio of the large mononuclear cells in the lymph to those in the peritoneal fluid is relatively low.

This may indicate that the supply of the large mononuclears is not immediately restored or that these cells do not return to the thoracic duct once they have been passed into the serous cavity. However, the number of these cells that show the effects of soaking in the fluid, as evidenced by loss of definite outline, loose chromatin, and pale staining, would seem to indicate that many of them are returned to the thoracic duct. Although the percentages of the large mononuclear cells in the lymph in my animals varied greatly, they did not go beyond the limits found in normal conditions.

Polymorphonuclear neutrophils constituted from 1.1 per cent. to 5.5 per cent. in this series of counts. This ratio is higher than the standard adopted, although Rous shows one instance of 11 per cent. with apparently only a slight admixture of blood. However, in my animals the number of polymorphonuclears apparently bears no relation to the other features of the condition. It would seem, then, that whatever may be the number of this type of cells in the lymph of the thoracic duct, it is not determined by the number furnished to the blood, or by the number removed from an effusion

containing a large number of these cells. This may be taken as another indication that the thoracic duct is not a highway for the polymorphonuclear neutrophils.

The relation of the *eosinophils* to the process under study appeared to be no greater than that of the polymorphonuclear cells just considered. My ratios ranged from 0.4 to 3.7 per cent.

The number of *transitional cells* in the lymph of my animals was much greater than was anticipated in view of what has been stated regarding their presence in the lymph of normal dogs. Their numbers range from 1.09 to 8.5 per cent. It may perhaps be significant that in the lymph the increase precedes and accompanies the increase of the polymorphonuclear cells in the effusion. However, the percentage of the transitional cells in the lymph is too low for us to attribute the supply of these cells in the blood to the number received from the thoracic duct. The small number of the cells in the lymph may be merely indicative of the way in which the body draws upon all possible sources of supply in conditions such as have been induced here.

The total cell count per cubic millimeter is variable and in itself does not bear a definite relation to the process. If at times during the course of the process we could compare each case with itself before the process began, perhaps more significant factors would appear. The rate of flow was as variable and as inconsistent, apparently, as the total cell count.

In considering all of these points it must be remembered that many factors, which it is difficult to have exactly alike in each case, have a bearing upon the results. The size and activity of the animal, the nature of the food, and the time it is taken are some of these factors.

In addition to the experiments just reported, others were made. One was suggested by a report of Opie[3] in which in dogs an effusion into one pleural cavity was secured by injecting turpentine into the other. This result was obtained only occasionally. To ascertain if the site giving rise to the effusion is identical with the field of irritation, I opened the peritoneal cavity of a dog, under chloretone anesthesia, and holding the intestines upward toward the diaphragm,

[3] Opie, E. L., *Jour. Exper. Med.*, 1907, ix, 391.

I placed a piece of gauze saturated with turpentine in the right inguinal region. Gauze was used rather than the free turpentine so as to prevent, as much as possible, any spread of the irritant. Holding the irritant in this position a quantity of fluid (fifteen to twenty cubic centimeters) collected within an hour, and it was plainly evident that portions of the serous membrane far distant were pouring out the fluid as well as the field locally irritated. This indicates that some nervous mechanism probably takes part in the process, and this explanation may also hold for the observations of Opie relative to the two pleural cavities.

The disposition of pigment placed within the peritoneal cavity was also considered in this connection. From the extensive investigation by Starling and Tubbey[4] it is concluded that soluble coloring matters are absorbed to the greatest degree from the serous cavity directly into the blood-vessels, the urine showing the color earlier than the lymph in the thoracic duct.

The method by which insoluble pigment is disposed of, and the route the pigment takes have also received much attention. The method established by von Recklinhausen[5] has been modified by more recent observers so that now we may consider that there are at least two general ways by which pigment is removed; namely, (1) through the agency of phagocytes, and (2) by the mechanical aspiration of the substance into the lymph channel by the respiratory movements. This subject, based upon the histology of the tissues concerned, is completely discussed by MacCallum.[6]

In my experiments I desired to see if the phagocytic cells followed the channels of the thoracic duct, but I failed to find a single polymorphonuclear cell containing pigment in the lymph from the thoracic duct. An occasional mononuclear enclosed a few granules of the red pigment previously injected into the cavity. However, pigment in appreciable quantities was found in the retroperitoneal and mediastinal glands. This would indicate that the phagocytes take a shorter and more direct route in depositing the foreign substance in the glands.

[4] Starling, E. H., and Tubbey, A. H., *Jour. Physiol.*, 1894, xvi, 140.
[5] von Recklinhausen, F., *Virchows Arch. f. path. Anat.*, 1863, xxvi, 172.
[6] MacCallum, W. G., *Bull. Johns Hopkins Hosp.*, 1903, xiv, 105.

CONCLUSIONS.

Injection of oil of turpentine into the peritoneal cavity of a dog calls forth immediately an exudate of fluid from the surrounding tissues. The amount of fluid reaches the maximum on the third day and has practically disappeared on the fifth day.

The cell content of this fluid is very small at first, but increases rapidly. The type of the predominating cells also changes. In the early exudate the small mononuclear cells are numerous and the large mononuclears few. Later the number of large mononuclear cells is increased and ultimately the polymorphonuclear cells preponderate. Various forms of atypical cells also occur.

Much of the fluid and many of the cells are removed by way of the thoracic duct. The counts of the cells in the thoracic duct and the estimates based on these indicate that the duct does not remove all of the fluid or cells from the peritoneal cavity. Much fluid is probably taken back directly into the blood, as are many of the cells. Some of the cells make their way to the lymph nodes, while many perhaps undergo complete autolysis in the serous cavity.

The polymorphonuclear cells do not enter the thoracic duct in great numbers. Examination of the lymph from the thoracic duct in the case of my dogs showed the types of cells that are usually found there.

The variation in small mononuclear cells is so related to the cell content of the peritoneal effusion as to indicate that the supply in the blood is maintained from this source. The form and staining qualities of the cells indicate that many of the small mononuclear cells are returned to the thoracic duct.

The ratio of polymorphonuclear cells present in the lymph bears no definite relation to the other features of the process. The transitional cells were increased in number and in their ratio to other cells. The increase in the transitional types accompanies an increase in large mononuclear cells and a decrease in small mononuclears.

None of the atypical forms of cells found in the effusion were seen in the lymph.

The number of eosinophils is without apparent relation to the other features of the process.

No polymorphonuclear cells containing the pigment injected were found in the lymph of the thoracic duct, and the number of mononuclear cells containing pigment was small. Much pigment was deposited in the lymph nodes. Detailed and definite conclusions as to the relation between the cells of the lymph and those of the effusion cannot be arrived at satisfactorily without repeated observations on the same animal.

Dr. Warthin examined many of the smears from these cases and frequently controlled the conclusions regarding the various types of cells. Dr. P. F. Morse assisted me frequently with the operative procedures and with the routine counting of the cells.

CHANGES IN THE TISSUE SURROUNDING A GROWING TUMOR AND THE SIGNIFICANCE OF THE "PRECANCEROUS STATE."*

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PLATES 11 AND 12.

One of the characteristics very frequently encountered in the early development of a malignant tumor is a certain degree of abnormality in the organs and tissues surrounding the growth. This condition very early attracted the attention of pathologists and for purely technical reasons the subject was studied almost exclusively on the epithelioma of the skin. The epithelial layer of skin is supported by subepithelial connective tissue, and since any change in the latter resembles an inflammatory condition, the impression was created that a developing malignant growth is surrounded by newly formed inflammatory connective tissue.

Ribbert (1), Bonney (2), Borrmann (3), and others consider this condition of paramount importance in the genesis of cancer and believe that the changes found in the connective tissue always precede the formation of the tumor. As the subject is stated in recent writings, a "precancerous state" in the surrounding tissue is essential for the formation of a cancer. von Hansemann (4) and his followers, on the other hand, hold that the connective tissue changes are secondary to the formation of the tumor and are influenced by the latter.

In the material derived from human cancer one usually sees the tumor and the changed surrounding tissue side by side, and it is impossible to declare with certainty which condition precedes and acts as a causative agent on the other. The solution of the problem

* Conducted at the expense of the George Crocker Special Research Fund. Read before the meeting of the American Association for Cancer Research, April 3, 1912. Received for publication, April 22, 1912.

may be expected only from an experimental study by the aid of which we may create artificially either an abnormality in an organ or the growth of a malignant tumor. In such experiments we may cause at will either the changes in the tissues to precede the growth of the tumor or the growth of the tumor to precede the changes in the tissues.

In a recent investigation the writer (5) succeeded in producing artificially a true "precancerous state," *i. e.,* an abnormality within an organ of the white rat which favored the subsequent growth of an inoculable cancer. The following is a brief statement of the experiments.

The work was conducted with an adenocarcinoma of the white rat described by Flexner and Jobling (6). This tumor grows readily in the rat when inoculated subcutaneously or when introduced into any of the parenchymatous organs with the exception of the testicle. In the latter the inoculation does not succeed. But when an abnormality is created within the testicle by an injection of two to three minims of Scharlach R-oil or by an injection of an emulsion of ether in water, then a subsequent inoculation with the tumor is successful. Thus an instance is found experimentally where a change in the tissue of an organ is absolutely essential to the success of a subsequent inoculation of a tumor, since the latter does not grow in the normal organ. In other words the creation of a "precancerous state" is indispensable for the success of the development of the inoculated tumor.

It is important to note here that the "precancerous state" created in this instance does not consist primarily of an inflammatory formation of new connective tissue. The growth of the inoculated tumor was influenced by the degenerative changes in the parenchymatous cells of the testicle and not by the newly formed connective tissue, as would have been claimed by Ribbert's school. The connective tissue formation could have been very profuse, but the tumor would not grow while the parenchymatous cells remained in a fair state of preservation.

But from these experiments the conclusion cannot be drawn that in every case the growth of tumors is aided by the changed surrounding tissues. Indeed, the experiments to be described later will show that instances occur in which no relationship exists between the growth of the tumor and the changes produced in the surrounding tissue.

The aim of the present investigation was to determine experimentally whether an abnormality in the tissue surrounding a growing tumor may be created under the influence of the tumor and subsequently to its development. The following method was employed.

INOCULATION OF TUMORS INTO MUSCLE AND LIVER.

A study of intracellular fatty infiltration affords the best means for detecting slight changes within a cell. Any impairment in the normal metabolism of the cell causes an increased accumulation of its fat contents. The question whether the accumulation of fat within the cell is an indication of a true degeneration, or whether it is due simply to nutritional causes, is of no consequence for the subject under consideration. The main point is that any undue accumulation of fat droplets in a cell is an indication of the beginning of a cell injury.

In order to detect the possible fatty changes in the tissue surrounding a growing tumor the following series of experiments were undertaken. A rat sarcoma was inoculated into the livers and muscles of a number of perfectly normal rats. At various intervals after the inoculations, the grafts were excised together with some of the tissue surrounding them and the excised pieces were placed in a 5 per cent. solution of formalin. Twenty-four hours later the tissues were cut by means of a freezing microtome and stained with Sudan III and hematoxylin. The examination of the specimens showed that while most of the tissue appeared normal, there was a zone of cells in the immediate neighborhood of the growing tumor, which was filled with an excessive amount of fat.

Figure 1 shows the condition in the liver in which a sarcoma was inoculated, and in figure 2 are seen a number of muscle fibers surrounded everywhere by tumor cells. Most of the muscle fibers are filled with fat. Although some of the liver cells and some of the muscle fibers surrounding the tumor show no fatty changes, the fact remains that a great many of the neighboring organ cells appear to be abnormal. The tumor graft had been placed in perfectly normal tissue, but then under the influence, mechanical or chemical, of the proliferating tumor cells the tissue cells became impaired in their normal metabolism and, as a consequence of this, had degenerated. An abnormality was here induced in a tissue that was previously healthy, but the sequence of events in this experiment is different from that in the experiments with the testicle. While in the experiments with the testicle the abnormality had to be created by some artificial means before the tumor could be made to grow,

in the present experiments the changes in the tissue were caused by the growth of the tumor and developed subsequently to this growth.

If the name "precancerous state" is to be applied to the condition described in the testicle of the white rat, then it may be permitted to give to the condition described in the present experiments the name "postcancerous state."

INOCULATION OF TUMORS INTO ANIMALS TREATED WITH PHOSPHORUS.

The previous series of experiments have shown that the cells of the organ surrounding a growing tumor undergo a fatty infiltration before they disappear and are replaced by tumor cells. Undoubtedly this fatty change aids the growth of the tumor to a certain extent. This second series of experiments was undertaken in order to ascertain whether a similar fatty change induced artificially in the parenchymatous cells of the liver would enable a tumor to grow in an organ of an animal known to be resistant to the growth of the same tumor.

As is well known, phosphorus poisoning induces in the cells of the liver an abnormal accumulation of fat, which is followed by necrosis and then by the formation of new connective tissue. This condition induced in the liver may resemble cirrhosis, and this was another reason for performing this series of experiments, for cirrhosis of the liver is very frequently followed by the development of a primary carcinoma in the liver. Indeed, some authors (Ribbert (7), Wheeler (8), and Huguenin (9)) believe that the primary cancer of the liver originates from a cirrhosis. According to their ideas, the cirrhosis induces the formation of an adenoma of the liver, and the adenoma is then transformed into a carcinoma.

Our experiments were conducted as follows: An injection of two to three minims of a 0.25 per cent. suspension of phosphorus oil was made subcutaneously into rats which were previously shown to be immune against the inoculation of the rat sarcoma. The injection was repeated three times at intervals of three days. Four days after the last injection the sarcoma was inoculated into the livers of these immune rats. In none of the twenty animals operated upon did the tumor grow, though the fatty changes in the liver cells were a

great deal more pronounced than in the cellular zone of the liver surrounding the tumor growth in a normal animal.

SUMMARY.

An analysis of the results of the experiments reported in this investigation shows that the changes so frequently observed in tissues surrounding a growing tumor may be caused by different conditions. The development and growth of a malignant tumor depends upon a local interaction between tumor cells and organ cells. When the cells of a normal organ are capable of inhibiting tumor growth, then an impairment of the normal state of the parenchymatous cells of this particular organ is essential for the growth of the tumor. This "precancerous state" does not consist primarily of an inflammatory change in the adjacent connective tissue, as Ribbert and his followers maintain, but in a degeneration of the parenchymatous cells of the organ.

If, in another instance, the cells of the normal organs are unable to inhibit the proliferation of the tumor cells, then no preparation of the cells of the organ for the tumor is necessary, *i. e.*, no "precancerous state" is needed to enable the tumor to grow. On the other hand, the proliferating tumor cells injure normal cells, either mechanically or chemically, producing a condition that appears on superficial examination like that described as the "precancerous state." In reality, however, this condition is the resultant effect of the tumor growth and may be more correctly designated the "postcancerous state." In these conditions, then, von Hansemann's explanation of the phenomenon, and not Ribbert's, seems to be the correct one.

Of still greater importance is the fact demonstrated in the last series of experiments; namely, that the general condition of resistance or immunity to cancer growth exerts a greater influence on the organism of the animal than any of the local conditions described above. The local resistance of a testicle to tumor growth in a generally susceptible animal may be overcome, but if an animal is made generally immune to the growth of cancer, neither the animal as a whole nor a single organ or tissue in it can be made susceptible to the growth of the tumor.

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EXPLANATION OF PLATES.

Plate 11.

Fig. 1. To the left of the drawing is sarcoma tissue; to the right, liver tissue. Sudan III and hematoxylin. High magnification.

Plate 12.

Fig. 2. Muscle fibers surrounded with sarcoma cells. Sudan III and hematoxylin. High magnification.

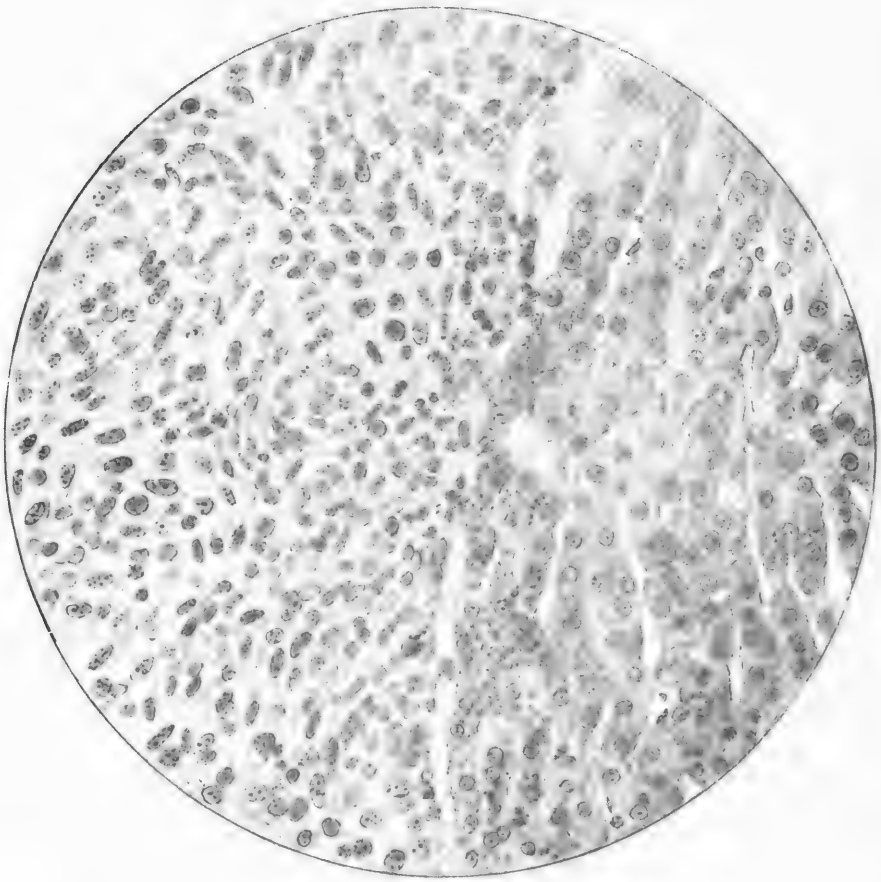

FIG. 1.

FIG. 2.

TUMOR INOCULATION INTO ORGANS AND THE ANALOGY BETWEEN HUMAN CANCER AND THE TUMORS OF WHITE MICE AND WHITE RATS.*

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PLATES 13–15.

The experimental study of inoculable tumors of white mice and white rats promises to be very fruitful, not only in elucidating the various phases in the pathogenesis of human cancer, but also in establishing correct methods for the specific therapy of the disease. The basic conception for this whole field of experimental cancer research is the most generally accepted idea that the tumors of white mice and white rats are analogous to human cancer.

Recently a grave doubt was cast upon the correctness of this idea. von Hansemann (1), in discussing his own and Wassermann's papers on chemotherapeutic experiments with inoculable tumors of white mice, emphatically declares that there is no analogy between these tumors and human cancer. He bases his opinion on the following differential characteristics between the two conditions. (1) Human cancers never reach as large a size in proportion to the body weight as do the tumors in animals. The only tumors that reach such a size in man are the benign growths, like the lymphangioma. (2) Animals do not suffer constitutionally from the tumors, *i. e.*, the state of cachexia is not observed. (3) The tumors in the mouse and rat appear as movable encapsulated nodules, which are easily removed surgically and do not recur after the operation. (4) A true metastasis does not take place in these animals. Conditions described as metastasis are simply multiple inoculations.

* Conducted at the expense of the George Crocker Special Research Fund. Read before the meeting of the American Association for Cancer Research, April 3, 1912. Received for publication, April 22, 1912.

When an emulsion of the tumor cells is injected subcutaneously, part of it may enter a blood-vessel and thus reach some distant part of the animal and form there a second tumor, which is, consequently, not a metastasis. (5) The inoculated tumors frequently retrocede, whereas human cancer does not. The apparent spontaneous cures described occasionally in human beings are due usually to a wrong diagnosis. (6) Morphologically the animal tumors resemble endotheliomata most nearly, and while it is not possible to prove absolutely the endothelial nature of the growths, the growths are not analogous morphologically to human carcinoma.

von Hansemann is one of the greatest authorities at present on the pathology of human cancer, and it is therefore imperative, in view of the great importance of the question for the future of experimental cancer research, to review the subject once more. The thesis the writer expects to sustain by the present investigation is that a white mouse or white rat bearing a tumor is suffering from a malignant disease which is analogous in every particular to human cancer.

SPONTANEOUS TUMORS.

In his discussion, von Hansemann apparently had in mind mainly the tumors artificially inoculated into previously healthy animals. In order to gain a true conception of the nature of the animal tumors, the analysis must begin with the spontaneously occurring tumors from which the material for the inoculation is taken.

The number of the morphologically different forms of these tumors that have been described in animals is nearly as great as the number of human cancers. Jobling (2) described cases of adenocarcinoma, cystadenoma, alveolar carcinoma, and sarcoma. Haaland (3) described carcinoma of the preputial gland, adenocarcinomata of the kidney and ovary, spindle-celled, round-celled, and polymorphous-celled sarcomata, melanoma, and fibromyoma of the uterus. It is evidently impossible to classify all these tumors as endotheliomata.

The best proof of the fact that these spontaneous tumors are malignant is the comparative frequency of the occurrence of metastases. Murray (4) in a study of sixty-eight mice with spontaneous tumors found metastasis in the lungs in twenty-seven cases, and in

the lymphatic glands in three cases. In studying twenty-six animals Jobling found metastases in five, and Haaland, in a study of 273 animals that had spontaneous tumors, found nodules clearly visible to the naked eye in the lungs of 38 per cent. of them.

Furthermore, most of the clinical characteristics of these tumors clearly indicate that the animals are suffering from a malignant disease. According to Haaland the average duration of life of the animals after the tumor has come under observation is about six weeks.

What is still more conclusive is the fact, also observed by Haaland, that in 54 per cent. of the animals with spontaneous tumors, the tumor recurred after an apparently radical operative removal. The same phenomenon was observed by Clunet (5). There can be no doubt, therefore, that a spontaneous tumor in a white mouse or white rat is malignant and consequently not only morphologically but also biologically analogous to human cancer.

It is impossible to define what is to be considered a condition of cachexia in these animals. The fact, however, that the animals die in the short period of about six weeks after the tumor is discovered, shows that the general health of the animal must have been deeply affected by the growth of the tumor.

INOCULATED TUMORS.

Tumors artificially reproduced through inoculation in previously normal animals are of greater importance for the experimental study of cancer than the spontaneous tumors. The reason is that the latter are comparatively rare, while inoculated tumors may be obtained in any desired number. These artificial tumors apparently seem to differ widely both from the spontaneous tumors of the same animals and from malignant growth in man. Although the material used for the inoculation is taken originally from a spontaneous tumor, *i. e.,* from an animal suffering from a malignant growth, the tumor that develops in the new host seems to be of a more benign character.

The transfer is usually made by a subcutaneous inoculation. As a result of this inoculation there forms under the skin a growth which may reach a very large size. In the majority of cases this

tumor is circumscribed, movable, and encapsulated. It usually does not infiltrate the surrounding tissue, it never infiltrates bone, peritoneum, or the pleura, and very seldom infiltrates the fascia and musculature underlying the skin. The skin itself, however, may become adherent to the tumor and ulcerate. Upon complete surgical removal, the tumor does not recur as does the spontaneous tumor. Thus the inoculated tumors apparently resemble benign growths rather than true cancers.

What is it that causes a tumor which was malignant in the first host to become apparently benign after transplantation? The recent studies of the writer (6) upon the behavior of inoculable tumors of white mice and white rats when implanted into various parenchymatous organs, convey the impression that the change in the behavior of the inoculated tumors is due to the method of transfer most generally employed. A subcutaneous inoculation may be the most convenient one for obtaining a sufficient amount of tumor material and is consequently useful for a number of different purposes. But in order to study the true behavior of the inoculated tumor and its influence on the general state of health of the new host, the inoculation should be made into the various parenchymatous organs.

INOCULATION INTO ORGANS.

For the inoculation into organs the writer employed the white rat mainly. Two tumors served for the inoculations: a spindle-celled sarcoma of the white rat, and an adenocarcinoma described by Flexner and Jobling (7). The tumors were inoculated into the brain, testicle, kidney, spleen, and liver. In the beginning of the investigation the organs were opened, the piece of tumor was placed inside, and the incision in the organ was closed with a suture. Subsequently the method was simplified. The organ was reached through an abdominal or lumbar incision, or by trephining the skull to reach the brain, and then a small piece of the tumor was placed in the center of the organ by means of a trocar needle. The bleeding even in the liver was minimal and ceased very soon. The abdominal or lumbar incision was then closed by a silk suture. The trocar needle does not cause any permanent injury to the organ.

When the tumor placed inside an organ fails to grow, it is with

difficulty that one can notice any change in the region where the tumor graft was placed, either by gross inspection or by microscopical examination. Consequently, whatever local change is found in the region of the organ surrounding the growing tumor is caused not by a mechanical injury through the operation, but by the interaction between the organ cells and tumor cells.

In order to compare the effects produced by inoculating a tumor into one of the organs of a susceptible animal, with the effects seen in human beings suffering from cancer, it is well to recapitulate the salient characteristics of malignant tumors. These characteristics are: (1) rapidity of growth; (2) peripheral extension, lack of capsule, and invasive infiltration of the surrounding tissue; (3) tendency to develop metastases and to recur after removal; and (4) cachexia and clinical malignancy. In the following pages a separate analysis will be made of these characteristics.

Rapidity of Growth.—If a white rat is inoculated successfully with either of the two tumors used in this investigation, it lives only ten to twelve weeks. The tumor produced by inoculation into an organ does not usually grow as large as that produced by a subcutaneous inoculation, but the number of days the animal survives is about the same after either form of inoculation. Apparently the large size which the tumors reach after a subcutaneous inoculation is not due to a peculiar character of the tumor cells, but to the fact that the subcutaneous tissue in these animals is very loose and consequently offers no resistance to the expansion of the tumor. It is much easier for the growing tumor to separate the skin from the underlying fascia than to invade the fascia.

Invasive Growth.—As stated above, when a tumor is inoculated subcutaneously it finds space for its peripheral extension in size by separating the skin from the superficial fascia and it usually does not invade the surrounding tissue. On the other hand, when a tumor is inoculated into an organ it does not increase its size by separating the parts of the normal organ tissue, but actually invades the latter and replaces it by tumor tissue.

Figure 1 shows a white rat with the abdominal wall removed and one lobe of the liver turned over; the other lobe shows in the center an inoculated sarcoma. The tumor apparently destroyed and re-

placed part of the liver tissue. Figure 2 shows two spleens inoculated with sarcoma. In the center of one spleen a small tumor growth is seen, but nearly all of the other spleen is replaced by a large tumor, the remains of the organ being noticed only at its two ends.

In some animals the tumor stays within the organ and the latter remains as freely movable in the abdomen as before the inoculation. In others the tumor invades and partly destroys the tissue or impairs the function of a neighboring organ; for instance, the stomach is injured when the inoculation is made into the liver or spleen. The autopsy findings in such an animal are analogous to those seen at the autopsy of a human being in which death was due to cancer in the peritoneal cavity.

This invasion of the neighboring organs cannot be ascribed in the present experiments to a multiple inoculation at the time of the operation, a criticism which was offered by von Hansemann to the experiments of Citron (8), who introduced into the wall of the stomach a thread permeated with a tumor emulsion. Here, apparently, there was a possibility for multiple inoculation. In the experiments of the writer, however, a solid piece of the tumor was introduced into the substance of the organ, and when the trocar was withdrawn, great care was taken to have the inoculated piece completely surrounded by the organ.

The microscopical appearance of the early stages of a sarcoma graft placed into the brain, testicle, or kidney was described by the writer in a former communication (6). The sarcoma cells infiltrate diffusely and replace the substance of the organ. In the present study similar experiments were performed with both sarcoma and carcinoma, the inoculations being made into the spleen, liver, and kidney.

Figure 3 shows the diffuse growth of a carcinoma in a spleen, and figure 4 the growth of a carcinoma inoculated into a liver. Carcinoma cells are seen to be growing diffusely between normal liver cells. Figure 5 shows an inoculation of a carcinoma into a kidney. The field shows kidney tubules surrounded everywhere by tumor cells. It is very clear that in this region most of the normal kidney tissue is replaced by the tumor. Thus it is evident that the invasive

infiltrating growth of an inoculable tumor implanted into a parenchymatous organ is similar to the growth of cancer in human beings. That the tumor behaves differently when inoculated subcutaneously is due then entirely to the nature of the tissue into which it is inoculated.

Formation of Metastases.—von Hansemann claims that a true metastasis was never described in inoculated tumors. Whenever a secondary tumor occurred in an animal in a region distant from the primary subcutaneous inoculation, it was due to the fact that part of the tumor emulsion used for the subcutaneous inoculation entered a blood-vessel, was transported to a distant part, and there formed a second tumor. The investigations of Levin and Sittenfield (9) do not bear out this assumption.

In the first place, even upon a direct injection of a tumor emulsion into the vascular system, the success of the formation of tumor nodules depends apparently upon the character of the tumor. While metastatic nodules were formed after an injection of an emulsion of carcinoma cells, similar experiments with sarcoma gave completely negative results. Furthermore, an inoculation of a solid piece of carcinoma into the bone marrow gave metastasis in the lung. The objection may be raised in regard to these experiments that a part of the inoculated piece of the tumor may have been torn off and carried readily from the bone marrow into the general circulation. The experiments of the writer given below seem to be free from all these objections.

Levin and Sittenfield did not succeed in producing metastases with rat sarcoma either by intravenous injection or by inoculation into the bone marrow. Of hundreds of animals inoculated subcutaneously with this sarcoma, metastasis was found only once and in this instance it was in the liver.

In the present series of experiments the sarcoma was inoculated into the livers of twenty rats, and the animals were kept alive for about four weeks. Metastasis was found in the lungs of three animals. The same sarcoma was inoculated into the spleens of twenty rats. Two of these animals developed metastases in the lungs and one in the liver.

Figure 6 shows one of the metastases in the lung. In all these

animals a solid piece of tumor was placed by means of a trocar in the substance of the liver or spleen. It is impossible then to suppose in these cases that the secondary nodule was formed at the time of inoculation. Apparently the metastatic nodule in the lung or liver was formed subsequently to the development of the primary tumor. Consequently the mechanism of the formation of a metastasis following an inoculation of a tumor into an organ is identical with that of the formation of metastases in spontaneous animal tumors and in human cancer.

Cachexia and Clinical Malignancy.—It is hardly possible to define the condition in a white rat or white mouse which would be identical with the cachexia in human beings, for the peculiar color and general condition of the skin, which is one of the main characteristics of the condition in human beings, cannot be obtained in these animals. But as was stated above, animals in which the inoculation was successful, do not survive the inoculation longer than about twelve weeks, and this is certainly direct clinical proof of the malignancy of these tumors. The following series of experiments gives further clinical proof of malignancy.

The writer (10) reported recently that the Flexner-Jobling carcinoma of the white rat does not grow when inoculated into a normal testicle, but it does grow in testicles which were treated previously with Scharlach R-oil or with ether water. Twenty-two animals in all showed a positive growth of the tumor. The testicles of these animals were removed surgically. During the operation the testicles did not appear to be adherent to the adjoining tissue nor were any small tumor nodules observed in the vicinity of the testicles. But in seven of these animals a secondary tumor developed near the operative field. The removal of these tumors had been as radical as any surgical operation for human cancer, but apparently microscopic groups of tumor cells remained in the neighborhood of the operation and a local recurrence took place.

SUMMARY.

The analysis of the experiments described above indicates that tumors of the white rat or white mouse inoculated into parenchymatous organs acquire a different biological character from those

inoculated subcutaneously. The latter are a great deal more benign in their behavior than human cancer or spontaneous tumors in the same species of animals. Tumors inoculated into organs, on the other hand, are quite identical in their biological behavior with the malignant tumors of animal and man. A conclusion must then be drawn, even *a priori,* that the method of inoculation into organs is a very important aid in the experimental investigation of cancer. It is true that the method is a great deal more complicated and time-consuming than the ordinary subcutaneous inoculation.

The subcutaneous method is satisfactory for a number of cancer problems. One of these is the study of general susceptibility and resistance of the organism of the host to the inoculation of the tumors, and this is a subject of paramount importance in cancer research. On the other hand, the investigations of the writer (10) have shown that an animal may be susceptible to a subcutaneous inoculation of a certain tumor and resist the inoculation of the same tumor into the testicle. Undoubtedly this method of inoculation will reveal the existence of a number of other phenomena.

The discovery of specific therapeutic measures is certainly the greatest problem in cancer research. A great deal of work has been done already on the subject, and the latest investigations of Wassermann on the chemotherapy of experimental tumors seem to be of great promise. But here also the therapeutic methods must be tried on animals in which the inoculations of tumor cells have been made into parenchymatous organs before the growths thus treated will have any analogy to human cancer.

In this connection one must bear in mind the fact that all the empirical so-called specific cancer remedies, which are continually being devised, are usually successful in treating localized skin cancers and fail utterly in the malignant growths of the internal organs. It is comparatively easy to produce a localized necrosis and softening in a circumscribed growth of the skin and subcutaneous tissue, but whether the same result will be produced on a diffuse and better nourished tumor growing inside of a parenchymatous organ cannot be decided *a priori.* To determine this it is necessary to have experimental proof on animals in which the tumor was inoculated into organs.

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EXPLANATION OF PLATES.

PLATE 13.

FIG. 1. A white rat with the abdominal wall removed and one lobe of the liver turned over. The left lower lobe shows in the center an inoculated sarcoma.

FIG. 2. The top figure shows a spleen with a small sarcoma growth in the center. The second drawing shows a spleen, nearly all of which is replaced by a large tumor. At both ends can be noticed the remaining parts of the spleen.

FIG. 3. The center shows a diffuse growth of a carcinoma surrounded everywhere by normal spleen tissue. Low magnification.

PLATE 14.

FIG. 4. The top of the drawing shows the growth of a carcinoma, which invades the liver. High magnification.

FIG. 5. A diffuse growth of a carcinoma surrounding four kidney tubules. High magnification.

PLATE 15.

FIG. 6. To the left of the drawing is a metastatic sarcoma nodule in the lung. To the right is normal lung tissue. Low magnification.

FIG. 1.

FIG. 2.

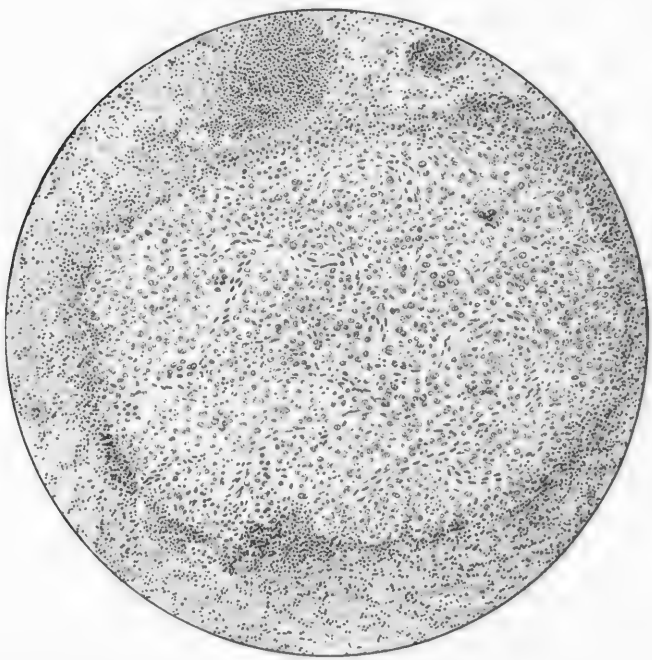

FIG. 3.

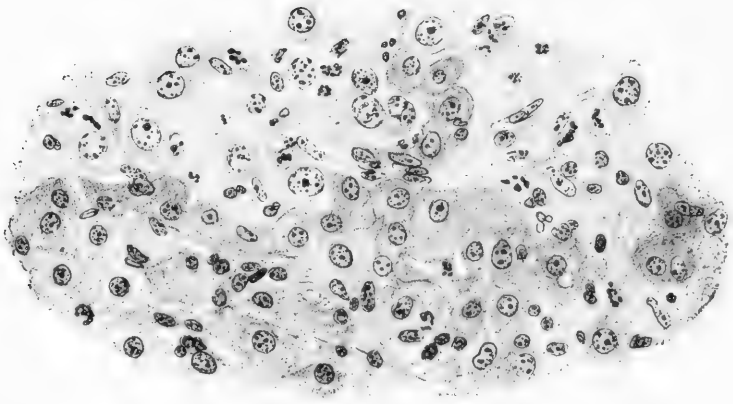

FIG. 4.

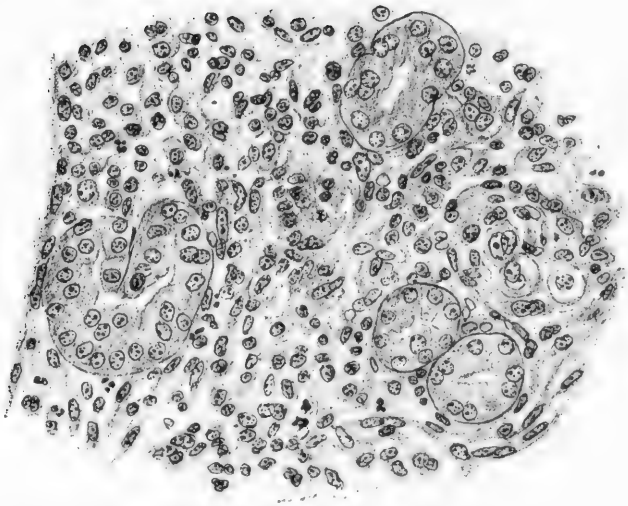

FIG. 5.

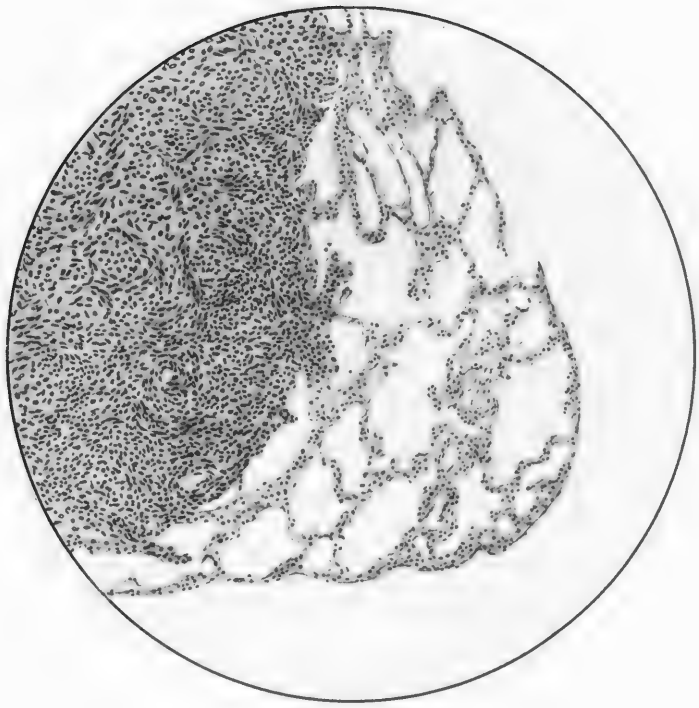

FIG. 6.

PURE CULTURES OF CELLS.*

By ALEXIS CARREL, M.D.

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The cells surrounding a fragment of tissue growing *in vitro* nearly always belong to several different kinds of tissues. It is important to isolate and cultivate a pure strain of cells of a determined type. I have attempted, therefore, to develop a technique by which pure cultures of cells might be obtained.

In its essentials the technique consists in isolating the cells of the selected type and in propagating them by repeated passages from medium to medium.

During the first few days of the growth of a culture of tissues, it was generally impossible to isolate cells belonging to a definite type. When the culture had undergone several passages, however, the cells grew more actively and spread over a larger area of the medium. Often a large group of cells belonging morphologically to one type could be seen growing in a thin layer. By careful microscopical examination it could be ascertained that no cells of a different type were present.

Then the part of the plasmatic jelly which contained the selected cells was isolated by cutting it away from the remaining part of the culture with a sharp cataract knife. The little film containing the cells was washed for one or two minutes in Ringer's solution and was then placed in a new medium. The new medium was composed of two parts of hypotonic plasma and one part of embryonic extract. A second microscopical examination showed that all the cells placed in this medium belonged to the type selected.

After the cells had been allowed to multiply for two or three days they underwent a second passage. They were allowed to grow again and subsequently underwent several passages. Generally after a few passages the cells grew together, forming a dense tissue.

* Received for publication, April 29, 1912.

The washings and passages were repeated as often as was necessary, and in this way it was possible to study the morphological changes in cells belonging to a known strain.

The experiments were carried out with ameboid and round connective tissue cells of the chick. The primary cultures were obtained from cultures of connective tissue, which were more than two months old. The old cultures were selected because it seemed that these cells had adapted themselves to their new form of life and that they could stand the washings and the passages better than the cells contained in the younger cultures. All the experiments in which the cells were taken from cultures more than sixty days old were successful. Two experiments only will be described.

PURE CULTURE OF AMEBOID CELLS.

Experiment 1. Primary Culture.—On March 20, 1912, a fragment of plasma at the peripheral part of a culture of heart sixty-three days old was resected. The fragment contained large ameboid cells only. Each cell could be seen individually. No round cells or spindle cells were present. The fragment was washed and put in a new medium. On March 21 no ameboid cells had passed into the new medium, but the number of cells contained in the old fragment of plasma had markedly increased.

First Passage.—On March 21 the culture was washed in Ringer's solution and put in a new medium. On March 22 the cells contained in the old plasma had increased so much that a real tissue was formed, but no ameboid cells were in the new plasma.

Second Passage.—On March 25 the area covered by the cells had become very large. The culture was therefore divided into two fragments (culture 1196–1 and culture 1196–2).

1. *Culture 1196–1.*—On March 26 the original fragment of plasma was surrounded by a large number of active ameboid cells. On March 28 the area covered by the cells had more than doubled. It was divided into two pieces and washed.

Third Passage.—One of the pieces was put in new plasma on March 28. On March 30 the culture had increased greatly in size and was again divided into two parts and washed.

Fourth Passage.—One of the pieces was put in new plasma on March 30, and grew abundantly.

On April 3, 6, 9, 12, 13, and 15, the culture underwent its fifth, sixth, seventh, eighth, ninth, and tenth passages. During that period, the growth, which was very luxuriant, was composed only of ameboid cells, and these were similar to those of the original culture.

After April 18 there was a very abundant growth of ameboid cells. On April 22 the culture was divided into two parts, and then underwent its twelfth

passage. The cells grew rapidly and on April 25 a thirteenth passage was made. On April 26 the cultures were composed of a mass of dense tissue, from the peripheral part of which radiated many ameboid cells.

2. *Culture 1196–2.*—On March 26 large ameboid cells, disposed in short columns, grew in the new medium. On March 28 the old plasma was surrounded by an immense number of cells covering a large area. The culture was divided into two parts and washed.

Third Passage.—On March 28 one of the parts was put in a new medium, and a very large number of ameboid cells, the pseudopodia of which were very short, grew in the new plasma. On April 1 the culture was divided into two parts.

Fourth Passage.—On April 1 one of the parts was put on a piece of silk veil and in a new medium. On April 2 the ameboid cells had covered a large area of the veil which was then divided into two parts.

Fifth Passage.—On April 2 one of the parts was put in new plasma, and on April 4 thick columns of ameboid cells had grown into the new medium from the periphery of the silk veil.

On April 5, 6, 9, and 12, the culture underwent its sixth, seventh, eighth, and ninth passages. The plasma contained in and around the silk network became denser, and the growth of the cells slower. After the tenth passage on April 15 chains of elongated cells appeared in the new plasma. No ameboid cells were observed.

On April 17, 20, 23, and 26, the culture underwent its eleventh, twelfth, thirteenth, and fourteenth passages. The growth was very slow and was composed only of polygonal and fusiform cells. No ameboid or round cells could be seen.

PURE CULTURE OF ROUND CELLS.

Experiment II. Primary Culture.—On April 1, 1912, a culture of connective tissue that had grown very actively for seventy-two days was selected. The central part was composed of a dense tissue surrounded by elongated cells. In the peripheral part of the plasma many round cells were scattered. With a cataract knife, the central portion was removed, leaving the crown of round cells. Microscopical examination showed that no spindle cells had been left. On April 3 the number of the round cells had increased enormously, but no fusiform cells had appeared.

First Passage.—On April 4 the culture was washed in Ringer's solution and put in a new medium. On April 5 the cells multiplied very rapidly; on April 6 a few spindle cells appeared; and on April 7 a large number of fusiform cells were scattered through the new medium.

On April 8 the culture underwent its second passage, and on April 9 the fusiform cells had increased greatly in number. The culture was divided into two parts on April 10.

After the third passage, on April 10, the new tissue increased in density.

On April 13, 16, 18, 22, and 24, the cultures underwent their fourth, fifth, sixth, seventh, and eighth passages. They were composed of a dense mass of tissue from which radiated a crown of elongated cells. There were no round or ameboid cells.

SUMMARY.

In experiment I a group of ameboid cells was isolated from a culture of cardiac muscle sixty-three days old, and cultivated in plasma. After several passages, they formed a dense tissue from which ameboid cells radiated. The culture was divided into two parts. The part cultivated in plasma alone kept its morphological characters and continued to produce ameboid cells. The part cultivated upon silk in plasma became modified; the cells lost their ameboid characters, and were transformed into large elongated cells which were united in chains, or interlaced to form a network.

In experiment II the round cells taken from a culture of connective tissue seventy-four days old multiplied rapidly. They transformed themselves into elongated cells and produced, after a few passages, a mass of dense connective tissue. From the tissue a large number of elongated cells were constantly growing.

In both experiments the tissues originated from the ameboid or round cells extirpated from cultures that were sixty-three and seventy-four days old respectively. These cultures were still growing actively thirty and forty days later; that is, more than one hundred days after the extirpation of the original fragments from the organism.[1]

These experiments show that from old cultures it is possible to isolate and propagate cells that belong to a definite type. A tissue, formed by a pure strain of cells, can be obtained in this way, and this new method may be of value in cytological investigations.

[1] The cultures were still living actively on June 2, 1912.

THE INFLUENCE OF ISOAGGLUTININS ON THE FINAL RESULTS OF HOMOPLASTIC TRANSPLANTATIONS OF ARTERIES.*

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(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

Plates 16 and 17.

Several investigators have reported that the functional results after homoplastic transplantation of arteries are excellent. But the microscopical examination of the transplanted segment, when made a long time after the operation, has given conflicting impressions as to the value of the transplantation. In several cases Carrel[1] found that after three to five months, not only the elastic framework, but even the muscular fibers of the median coat were in good condition.[2] Borst and Enderlen,[3] on the other hand, observed a disappearance of the muscular fibers and a substitution of the transplanted segment by the connective tissue of the new host, just as in heteroplastic transplantation. Yamanouchi also found a degeneration of the segment when this was examined seventy-four days after the operation.

It is, of course, possible that differences in the technique of these operations may be responsible for the lack of uniformity in the results. But it is also possible that biological differences in the two animals concerned in each experiment were in some cases so great that they prevented the survival of the transplanted pieces.

It is not yet known why homoplastic transplantation does not usually succeed as well as autoplastic. But in the experiments which

* Received for publication, April 23, 1912.

[1] Carrel, A., *Jour. Exper. Med.*, 1910, xii, 460.

[2] Capelle, A., *Berl. klin. Wchnschr.*, 1908, xlv, 2012. Capelle, Stich, Ward, and Wood had corresponding results, but their examinations of the segment were made within a somewhat shorter interval after the implantation.

[3] Borst and Enderlen, *Deutsch. Ztschr. f. Chir.*, 1909, xcix, 54.

are published here, I have attempted to solve part of the problem, and even if the result is a negative one, it may still be of some interest.

In animals of the same species normal hemolysins or cytolysins have not been found, which may explain the biological differences in the individuals, and be made responsible for the disintegration and death of the homoplastic transplanted piece of tissue. We may conceive of the development of immune bodies under these conditions, but there is no evidence of their existence. Agglutination is the only serological reaction that is normally present between the serum of certain individuals and the red blood cells of others. This phenomenon has been rather thoroughly studied during the last few years, and the importance of the isoagglutinins in practical medicine has been increased by the development of direct transfusion of blood from man to man, as a routine measure.

Normal isoagglutinins of human red blood cells were discovered independently by Landsteiner[4] and Shattock in 1900, and Landsteiner showed that all specimens of human blood could be divided into three groups according to the way in which they agglutinate. To these three groups a fourth was added by other investigators.[5] Landsteiner then assumed that the four groups were explained by the existence of two different agglutinins, one of which was present in group 2, another in group 3, both in group 1, and none in group 4.

Epstein and Ottenberg[6] and von Dungern and Hirschfeld have discovered the interesting fact that the isoagglutinins are transmitted by heredity and follow the Mendelian law. Grouped isoagglutinins have been found also among lower animals. Ottenberg and Friedmann[7] found grouped agglutinins in rabbits and cattle.

I have tried now to determine if the isoagglutinins exert any influence upon the final results of homoplastic transplantation of arteries.

[4] Landsteiner, K., *Wien. klin. Wchnschr.*, 1901, xiv, 1132; *München. med. Wchnschr.*, 1902, xlix, 1905.

[5] Landsteiner, K., and Leiner, K., *Centralbl. f. Bakteriol., Orig.*, 1905, xxxviii, 548.

[6] Epstein, A. A., and Ottenberg, R., *Proc. N. Y. Path. Soc.*, 1908, viii, 117; *Arch. Int. Med.*, 1909, iii, 467.

[7] Ottenberg, R., and Friedmann, S. S., *Jour. Exper. Med.*, 1911, xiii, 531.

First, I tried to find isoagglutinins in dogs, but without success. The same result is reported by Hektoen.[8] After several fruitless experiments, that were hampered by the tendency of dog blood to lake, I began to work with cats.

The blood of forty cats was examined in four series, each of which contained ten animals, and the serum of each cat was tested against the red cells of all the other cats in the same group. In these experiments the technique described by Epstein and Ottenberg was used. One volume of a 5 per cent. suspension of erythrocytes in isotonic salt solution was mixed with two volumes of serum in capillary pipettes. The pipettes were incubated at 38° C. for two hours and then put in an ice box for twenty-four hours. Each series was in that way examined twice. The results are recorded in the following tables.

Blood Cells.	Serum. 1	2	3	4	5	6	7	8	9	10
1	—	—	—	—	—	+	—	—	—	—
2	—	—	—	—	—	+	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	+	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—
6	—	—	—	+	—	—	—	+	—	—
7	—	—	—	—	—	+	—	—	—	—
8	—	—	—	—	—	+	—	—	—	—
9	—	—	—	—	—	+	—	—	—	—
10	—	—	—	—	—	+	—	—	—	—

Blood Cells.	Serum. 11	12	13	14	15	16	17	18	19	20
11	—	—	—	—	—	—	—	—	—	—
12	—	—	—	—	—	—	—	—	—	—
13	—	—	—	—	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—	—	—
16	—	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—	—	—
18	—	—	—	—	—	—	—	—	—	—
19	—	—	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—	—	—

[8] Hektoen, L., *Jour. Infect. Dis.*, 1907, iv, 297.

Serum.

Blood Cells.	21	22	23	24	25	26	27	28	29	30
21	—	—	—	—	—	—	—	—	—	—
22	—	—	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	—	—	—	—
25	—	+	—	—	—	—	—	—	—	—
26	—	—	—	—	—	—	—	—	—	—
27	—	—	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	—	—	—	—
29	—	+	—	—	—	—	—	—	—	—
30	—	—	—	—	—	—	—	—	—	—

Serum.

Blood Cells.	31	32	33	34	35	36	37	38	39	40
31	—	—	—	—	—	+	—	—	—	—
32	—	—	—	—	—	—	—	—	—	—
34	+	—	—	—	—	+	—	—	—	—
35	—	—	—	—	—	+	—	—	—	—
36	—	—	—	—	—	—	—	—	—	—
37	—	—	—	—	—	—	—	—	—	—
38	+	—	—	—	—	+	—	—	—	—
39	—	—	—	—	—	—	—	—	—	—
40	—	—	—	—	—	—	—	—	—	—

The reaction was recorded as positive (+) only when, after macroscopical inspection, the agglutination was plainly seen on the first as well as on the second examination. In two or three cases the agglutination was very strong and occurred some minutes after the mixture had been made; but usually it was not marked until the next day; *i. e.*, after twenty-four hours in the ice chest.

As may be seen in the tables, the instances of agglutination are distributed among the cats without the slightest possibility of arranging the individuals in groups having the same characteristics.

In some of these cats a segment of the carotid artery (two centimeters long) of one was transplanted to the carotid of the other. In these operations I followed closely the technique described by Carrel. Fourteen transplantations were made; in five of them there was a positive agglutination test between the two animals, and in nine of them the agglutination test was negative. After the segment had been taken out, it was washed in Ringer's solution and then kept in vaselin at 38° C. until it was put in the new host, one half to three fourths of an hour later.

The operation and the convalescence in these fourteen cases were successful and uneventful. Infection and bleeding did not occur.

All of the fourteen transplanted segments were removed from the cat three months after they had been transplanted and were then examined microscopically. Of the five cats in which the agglutination test was positive, three had ideal functional results, and of the nine animals in which the agglutination tests were negative five had ideal functional results with good pulsation and no thrombosis of the artery. In the other six cases there was thrombosis of the artery and of the transplanted segment.

That thrombosis occurred in such a large percentage of the cases is not astonishing and is explained perfectly upon mechanical grounds. As the carotid artery in my cats was only one millimeter in diameter, the slightest stenosis or lack of approximation in suturing produced complete thrombosis of the whole lumen of the vessel.

I shall describe only the experiments in which the functional results were good, as it is not possible to draw conclusions of value from the microscopical examination of thrombosed arteries. Although the muscular fibers with which we are here especially concerned are exposed in thrombosed arteries to the blood serum of the new host and for this reason may show changes due to chemical influences or lack of proper nourishment, I have discarded them in these experiments since the muscular fibers in a thrombosed artery are inactive, and also are under abnormal conditions, both biologically and functionally.

INTERAGGLUTINATION POSITIVE.

Experiment 1.—January 25, 1912. From cat 25 a segment of the carotid artery 2 cm. long was taken and transplanted to the carotid artery of cat 22. The serum of cat 22 agglutinated the red cells of cat 25.

April 22. At the operation good pulsation was present in the carotid but there were some adhesions between the segment and the pneumogastric nerve. The segment was removed.

Microscopical Examination.—Adventitia and periadventitia were thickened, and richly vascularized. The median coat was of normal thickness, the muscular fibers and their nuclei were present in normal number and their appearance was quite normal. With Weigert's stain the elastic framework appeared normal. The internal coat was not thickened (figure 1).

Experiment 2.—December 14, 1911. A segment of the carotid artery of cat 1 was taken and transplanted to the carotid artery of cat 6. The serum of cat 6 agglutinated strongly the red cells of cat 1.

March 16. At the operation good pulsation was present in the carotid but the transplanted segment was bound by slight adhesions to the surrounding tissues. The segment was taken out.

Microscopical Examination.—The adventitia was thickened, richly vascularized, and contained several more lymphocytes than usual. The median coat was only half its normal thickness. All the muscular fibers and their nuclei had disappeared and the elastic framework layers were lying close to each other; the median coat contained nothing but elastic fibers. The internal coat was very much thickened (figure 4).

Experiment 3.—December 14, 1911. A segment of the carotid artery of cat 6 was transplanted to the carotid of cat 8. The serum of cat 6 agglutinated strongly the red cells of cat 8, and the serum of cat 8 agglutinated the cells of cat 6 only a little less strongly.

March 17, 1912. At the operation good pulsation was present in the carotid. There was no thrombosis, but adhesions were found between the segment and the internal jugular vein and the pneumogastric nerve. The segment was taken out.

Microscopical Examination.—The adventitia was thickened, and richly vascularized. The median coat was of normal thickness. In its outer layers the muscular fibers and their nuclei were normal in number and appearance. In its inner portion the fibers seemed a little atrophied and had taken only slightly the protoplasmic stain of Van Gieson. The nuclei had largely disappeared. There was no increase of the connective tissue; the elastic framework was normal; and the internal coat was slightly thickened.

INTERAGGLUTINATION NEGATIVE.

Experiment 4.—December 9, 1911. A piece of the carotid artery of cat 18 was transplanted to cat 19. Between these two cats the agglutination test was negative.

March 8, 1912. At the operation good pulsation was present in the carotid but there were adhesions to the vagus and the internal jugular vein. The segment was taken out.

Microscopical Examination.—The adventitia was considerably thickened, and consisted partly of thick hyaline connective tissue bundles. Around the small vessels, aggregations of lymphocytes were seen. The median coat was thin and flattened. Almost all of the muscular fibers and their nuclei had disappeared. The elastic framework layers were lying close to each other, and the internal coat was covered by a thin layer of amorphous fibrin.

Experiment 5.—January 4, 1912. A piece of the carotid artery of cat 4 was transferred to cat 9. The agglutination test between these two cats was negative.

April 3. At the operation good pulsation was felt in the carotid but there were adhesions to the surrounding tissue. The segment was taken out.

Microscopical Examination.—The adventitia was thickened and richly vascularized. The inner layers were sclerotic. The median coat was a little thinner than normally. In the inner portion the muscular fibers had disappeared. In the outer portion they were still present in almost normal number.

The fibers were atrophied, their nuclei were not very distinctly stained, and their outlines were not sharp. The elastic framework was normal. In the internal coat there were three to four layers of connective tissue cells; in other places the internal coat was not thickened.

Experiment 6.—January 3, 1912. A piece of the carotid artery of cat 11 was transplanted to cat 12. The agglutination tests between these animals were negative.

April 5. At the operation good pulsation was felt in the carotid but there were adhesions between the segment and the surrounding tissue. The segment was taken out.

Microscopical Examination.—The adventitia was much thickened and richly vascularized in the outer layers; the inner layers were partly sclerotic. The median coat was of normal thickness. In the outer layers there were a normal number of muscular fibers, but in the inner layers these had partly disappeared. The elastic framework was normal. The internal coat was a little thickened and had three to four layers of connective tissue cells.

Experiment 7.—January 9, 1912. A piece of the carotid artery of cat 14 was transplanted to the carotid artery of cat 15. The agglutination tests between these two animals were negative.

April 14. At the operation good pulsation was felt in the carotid but there were adhesions to the internal jugular vein and the pneumogastric nerve. The segment was taken out.

Microscopical Examination.—The adventitia was greatly thickened. The median coat was thin and flattened and consisted almost exclusively of the elastic framework, the muscular fibers having disappeared for the greater part. The internal coat was slightly thickened by the development of very thin elastic fibers.

Experiment 8.—January 11, 1912. A piece of the carotid artery of cat 24 was transplanted to the carotid artery of cat 26. The agglutination tests between these two animals were negative.

April 13. At the operation good pulsation was felt in the carotid but there were adhesions to the surrounding tissue. The segment was taken out.

Microscopical Examination.—The adventitia was thickened, but the median coat was thinner than normally. Many of the muscle fibers had disappeared; the elastic framework was normal, and the internal coat was a little thickened.

The sections of the arteries were stained with Van Gieson's stain, with Weigert's elastic tissue stain, and with hematoxylin and eosin.

Results.—In all of the transplanted arteries examined three months after transplantation, there was a thickening of the external coat and a persistence of the elastic fibers of the median coat. Further I found in most of the cases a more or less pronounced thickening of the internal coat.

Very often the external coat had thickened until it was three times its normal size. The new layers of connective tissue had the appearance of cicatrizing tissue; the external coat was somewhat infil-

trated by round cells and many small vessels were seen, in the walls of which lymphocytes were aggregated. The bundles of connective tissue were thick, and the inner layers were partly hyaline and sclerotic.

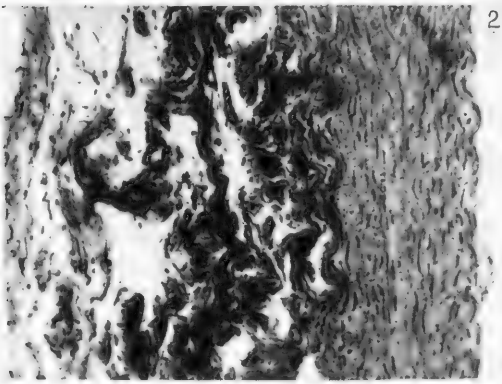
The elastic framework of the median coat was always found in good condition; splitting of the fibers or the formation of granules was not observed. The appearance of sections stained by Weigert's elastic tissue stain, however, was influenced by the presence or absence of the muscle fibers. If all of the muscle fibers had disappeared, the elastic fibers and layers were pressed together and lay closely to each other without any tissue between them. The median coat as a whole was very thin (figure 2). If, on the other hand, the muscle fibers were present, the elastic framework had quite a normal appearance (figure 3). In no case was a proliferation of cells of any kind, or a formation of new tissues within the median coat observed. In most of the cases the smooth muscle fibres of the median coat had partly disappeared.

There were some few features of interest. In the first group of transplantations (three experiments with positive interagglutination) the muscle fibers were apparently quite normal in appearance and number in experiment 1, and also perhaps to a less extent in experiment 3, while in experiment 2 these fibers had completely disappeared (figures 1, 2, and 3). Experiments 1 and 2 are within the same group and yet are at opposite extremes as far as the muscular fibers are concerned. Experiment 2 was the one in which the isoagglutination was strongest, but that this coincides with the most complete disappearance of muscular fibers must be considered accidental, because the other two experiments in the same group show muscular fibers in a very good condition.

In the second group (five experiments, in which interagglutination was negative) the results in the different experiments corresponded more closely. Here most of the muscular fibers, but not all had disappeared, and as a general feature it was observed that they had disappeared in the inner layers of media, while they were still present in the outer.

The thickening of the internal coat went parallel with the disappearance of the muscle fibers of the median coat; the strongest

1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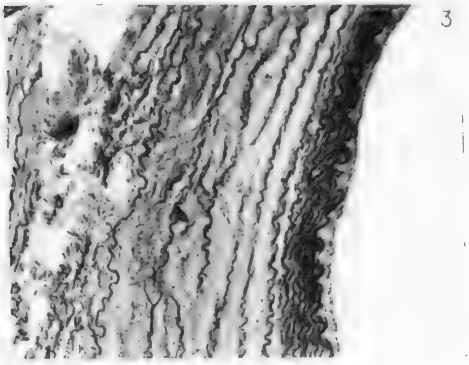
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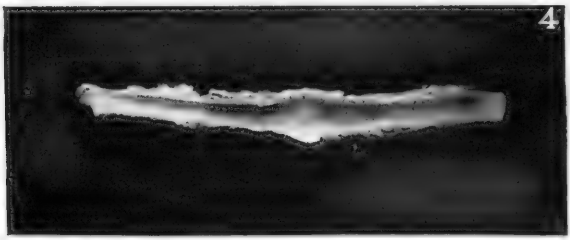
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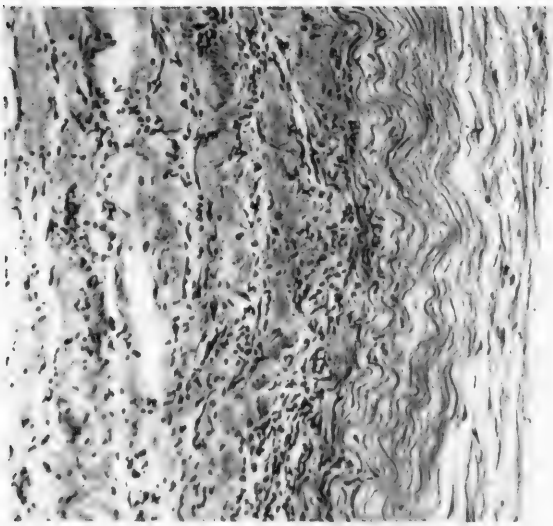
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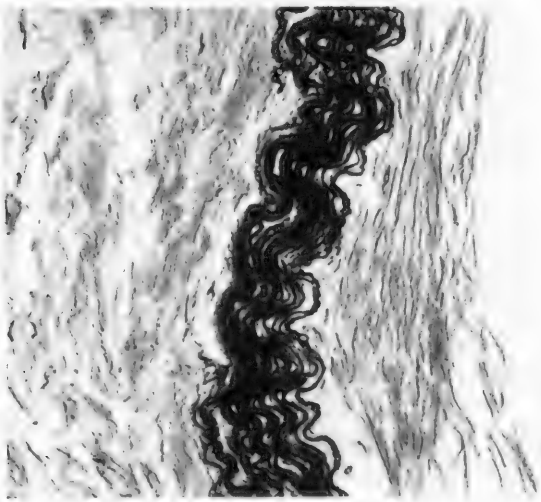
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increase was seen in experiment 2 where all of the muscle fibers had disappeared. In experiment 1 where all of the muscle fibers were present, the internal coat was thickened but little (figures 1 to 6).

CONCLUSIONS.

The results in the group in which there was interagglutination differ in no striking way from those obtained in the group in which there was no interagglutination. It may be concluded, therefore, that the presence of isoagglutinins is of no importance for the final results of the homoplastic transplantation of arteries. From the conflicting evidence of the survival of muscular fibers in one case and their disappearance in many other cases, it may be concluded further that between animals of the same species there are unknown biological differences that prevent, in most instances, the survival of homoplastic transplanted arteries, although a survival does occur in some cases.

EXPLANATION OF PLATES.

PLATE 16.

FIG. 1. Gross appearance after three months of a segment of a carotid artery transplanted from cat 25 to cat 22. Agglutination test positive.

FIG. 2. Transverse section of the segment. Internal coat not thickened The median coat has the normal number of muscle fibers and nuclei. The external coat is somewhat thickened. Van Gieson's stain.

FIG. 3. Transverse section of the same specimen. Internal coat slightly thickened here. Weigert's elastic tissue stain.

PLATE 17.

FIG. 4. Gross appearance after three months of a segment of a carotid artery transplanted from cat 6 to cat 1. Agglutination test positive. The suture lines can hardly be seen.

FIG. 5. Transverse section of the transplanted segment. The internal coat is very thick. The median coat is thin without muscle fibers. The external coat is very thick and infiltrated by round cells. Van Gieson's stain.

FIG. 6. Transverse section from the same specimen. Weigert's elastic tissue stain.

STUDIES IN GLYCOSURIA.

Second Paper: Glycosuria Following Anesthesia Produced by the Intravenous Injection of Ether.*

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In a previous study (1) regarding the nature of the glycosuria following the administration of ether by the respiratory method, the following points were made. 1. Glycosuria occurs in healthy adult dogs usually at the end of an hour and lasts from seven to twenty-four hours. 2. There is an accompanying hyperglycemia. 3. The source of the hyperglycemia is the liver, since a partial exclusion of this organ from the circulation by means of an Eck fistula prevents the occurrence of the hyperglycemia and the glycosuria. 4. Section of the splanchnic branches to the liver from the celiac plexus does not prevent the occurrence of the hyperglycemia and the glycosuria. This suggested that the hyperglycogenolysis associated with ether anesthesia could be caused independently of the participation of the splanchnics.

MacLeod (2) has shown for the glycosuria caused by asphyxia that, when the asphyxia is light, section of the splanchnics going to the liver entirely prevents the occurrence of glycosuria; but when the asphyxia is profound, then the section of the nerves fails to prevent glycosuria.

The question as to whether the hyperglycemia and the glycosuria that follow the administration of ether are due to an increase in the carbon dioxid content of the blood as in the glycosuria of asphyxia, or whether they are expressions of diminished oxidative power on the part of the tissues, was not investigated. Observation made at the end of the anesthesia, when the hyperglycemia was present, gave no evidence of the venous type of blood. But the only accurate

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method of settling this point would be, as Henderson (3) suggests, to analyze the gaseous content of the blood.

An attempt was next made to find some method of producing ether anesthesia without causing asphyxia. The intravenous route was considered promising and attempts were made to induce anesthesia by such a procedure. The results form the subject matter of this communication.

In the beginning, ether was introduced in varying concentrations in defibrinated blood, but this method was unsatisfactory because there resulted marked hemolysis and hemoglobinuria. Similarly when ether was introduced in blood serum these same disturbing factors occurred. Moreover, anesthesia was not complete.

The excellent method described by Rood (4) was then tried and was employed in all of our experiments. This observer describes a very satisfactory method for causing anesthesia. He found that a 5 per cent. solution of ether in normal saline made a uniform solution and that when this was introduced into the veins of a patient it produced a very satisfactory surgical anesthesia. In general, it required about eight ounces of the fluid to produce the state of anesthesia, and this could be maintained with as little as one pint per hour. Furthermore, hemoglobinuria was never produced and this made the problem of the study of the urinary findings much more satisfactory.

The method has been used on animals by several observers, but only within a year or two has it been applied to man (Burckhardt (5)). Since then it has been employed successfully in Germany, especially by Kämmand, and in England by Rood. For the more practical points regarding its surgical value I shall have to refer the reader to the paper of Rood (6). Suffice it to say here that in all of our dogs a satisfactory anesthesia could be obtained by this procedure. The amount of fluid necessary to produce anesthesia varied considerably with the size of the animal, ranging between 200 and 600 cubic centimeters. There was very little danger associated with administering the anesthetic and the animal came out rapidly. It was found in two cases, however, that, if too much of the solution was introduced rapidly when the animal was deeply anesthetized, death resulted speedily with paralysis of the respiration. The heart

continued to beat strongly for some minutes, but attempts at resuscitation failed to revive the animals. However, it must be said that with any reasonable care the anesthesia appears to be safe and effective.

EXPERIMENTAL PART.

Method.—Good sized, adult female dogs were chosen. In the majority of the cases they were placed on a meat diet. Before the beginning of the experiment the animal was catheterized and the urine obtained was tested for sugar and albumin. The jugular vein was then exposed under local anesthesia (0.1 per cent. cocain), and the ether solution was introduced directly into the vein by means of an apparatus modeled after that described by Rood. The solution was introduced at approximately the same rate in each experiment, about 150 cubic centimeters every fifteen minutes. The animals were catheterized every fifteen minutes, the amounts were recorded, and the specimens obtained were tested for sugar and albumin. In those experiments in which the blood sugar was studied, the technique employed was the same as that described in a previous paper (1).

At the conclusion of the experiments the animals were placed in metabolism cages and the urine collected for twenty-four hours was likewise tested for sugar and albumin. The qualitative test for sugar employed was always Fehling's, and for albumin the nitric acid and heat, and the acetic acid tests. Quantitative determinations of sugar were made by the Bertrand method (7). These experiments may be grouped into three general classes; (1) a preliminary series in which it was demonstrated that the anesthesia produced by the ether-saline method was accompanied by glycosuria; (2) a series in which the saline solution (0.9 per cent.) alone was introduced intravenously; (3) a series in which the change in the percentage of the blood sugar was studied in each of the above types.

Other experiments confirmed the findings in series I, but in order to avoid unnecessary repetition the protocols will not be given in detail. The more complete experiments show all of the important findings in a very satisfactory way. From these it appears that the production of anesthesia by the intravenous administration of an

SERIES I. PRELIMINARY STUDY.

Dog C 19.—Fox terrier, female; weight 23.5 pounds.

The animal was catheterized before the beginning of the experiment, and the urine was negative for sugar and albumin. A 5 per cent. solution of ether in 0.9 per cent. sodium chlorid was introduced into the right internal jugular vein. The amount introduced was 700 c.c.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	
15 min.	0	0	0	
30 min.	5 c.c.	+	0	Animal under surgical
45 min.	9 c.c.	+	0	anesthesia.
75 min.	26 c.c.	+	0	
90 min.	26 c.c.	+	0	Discontinued transfusion.

The urine collected for twenty-four hours amounted to 820 c.c. and contained 0.4067 gm. of sugar and no albumin.

ether-saline solution is accompanied by glycosuria. The clinical features of the anesthesia will be described later.

SERIES II. THE INTRAVENOUS ADMINISTRATION OF SALINE SOLUTION (0.9 PER CENT.).

Underhill and Closson (8) review the literature on salt glycosuria, and it may perhaps be permitted here to give briefly the important facts which these authors bring out in their paper in order that they may be compared with the findings in our series.

In 1871 Bock and Hoffmann (9) found that large amounts of a 1 per cent. solution of sodium chlorid injected into the arterial circulation in rabbits produced a diuresis which was followed by the appearance of sugar in the urine.

Külz (10) obtained similar results with sodium chlorid, sodium acetate, sodium carbonate, sodium valerianate, and sodium succinate.

Martin Fischer (11) found that when a solution of N/6 sodium chlorid is injected into the lateral vein of the ear of a rabbit at the rate of 75 to 100 c.c. every fifteen minutes, a polyuria makes its appearance in from ten to fifteen minutes and sugar appears in the urine about two hours after the beginning of the injection. When a more concentrated solution of sodium chlorid is used, N/2 sodium chlorid, sugar appears in the urine after 20 to 30 c.c. have been introduced. Solutions less concentrated than N/6 sodium chlorid fail to produce a glycosuria, although a polyuria may be observed. Furthermore, when glycosuria has been established by saline infusions, the excretion of sugar may be markedly lessened or entirely inhibited by substituting for the salt solution a mixture of sodium and calcium chlorid.

Underhill and Closson state: "The fact that sodium chlorid solution when injected intravenously induces polyuria and glycosuria; that the glycosuria appears more rapidly the greater the concentration of the salt solution; that

the excretion of sugar may be lessened or inhibited by substitution of a mixture of sodium chlorid and calcium chlorid; and that the introduction of the latter is followed by a temporary fall in the amount of urine excreted per unit of time, point to an increased permeability of the kidney as the factor involved."

Fischer observed later that if sodium chlorid solutions were injected into the arterial circulation, glycosuria ensued much more rapidly than when they were injected into the venous circulation. He formulates the conclusion that the mechanism of the production of salt glycosuria involves two factors, one, an action on the kidney causing diuresis, and a second, an influence on the diabetic center in the spinal bulb which causes glycosuria.

Underhill and Closson object that the above quoted deductions hardly seem conclusive, especially that there is not sufficient evidence for assuming a specific action of the salt on the diabetic center. They suggest that a more exact knowledge of the nature of the glycosuria occurring in each of the above types of sodium chlorid infusion could be arrived at by a study of the quantitative changes of the sugar content of the blood.

To put the matter briefly, Underhill and Closson found that associated with the intravenous injections of sodium chlorid there occurs a hypoglycemia during the time of appearance of sugar in the urine, whereas, when the sodium chlorid solutions were injected into the cerebral arterial circulation, the glycosuria is associated with a hyperglycemia but not with a polyuria. The increase in blood sugar that occurs later is referred by them to the influence of the accompanying asphyxia which inevitably leads to hyperglycemia and at times to glycosuria (12).

It was obviously of importance for us to ascertain whether the introduction of the normal saline solution was associated with a glycosuria. To establish this point several experiments were performed after the manner already described for the ether-saline injection.

Fresh 0.9 per cent. sodium chlorid (Baker analyzed, chemically pure) was prepared for each experiment and sterilized by heat before being introduced into the veins. The protocols of a few of the experiments follow.

Dog C 20.—Fox hound, female; weight 34 pounds. The animal was catheter-

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	
15 min.	7 c.c.	0	0	
30 min.	62 c.c.	0	0	
45 min.	258 c.c.	0	0	
1 hr.	135 c.c.	0	0	Introduction discontinued.
1 hr. 15 min.	98 c.c.	0	0	
1 hr. 35 min.	38 c.c.	0	0	
1 hr. 45 min.	39 c.c.	0	0	
2 hrs.	82 c.c.	0	0	

The urine collected for twenty-four hours amounted to 2,500 c.c. This contained no sugar and only a trace of albumin.

ized before the beginning of the experiment, and the urine contained no sugar or albumin. 1,700 c.c. of sterilized, freshly prepared 0.9 per cent. sodium chlorid solution were introduced into the right internal jugular vein.

Dog C 28.—Brindle bull; weight 22 pounds. The animal was catheterized before the beginning of the experiment, and the urine obtained was negative for sugar and albumin. 2,000 c.c. of sterilized, freshly prepared 0.9 per cent. sodium chlorid solution were introduced into the right internal jugular vein.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	
15 min.	3 c.c.	0	0	
30 min.	13 c.c.	0	0	
45 min.	35 c.c.	0	0	
60 min.	62 c.c.	0	0	
1 hr. 15 min.	75 c.c.	0	0	
1 hr. 30 min.	55 c.c.	0	0	
1 hr. 45 min.	50 c.c.	0	0	
2 hrs.	60 c.c.	0	0	
2 hrs. 15 min.	60 c.c.	0	0	
2 hrs. 30 min.	48 c.c.	0	0	Introduction discontinued.
2 hrs. 45 min.	110 c.c.	0	0	
3 hrs.	58 c.c.	0	0	
3 hrs. 30 min.	74 c.c.	0	0	
3 hrs. 45 min.	96 c.c.	0	0	

The urine collected for twenty-four hours after the experiment amounted to 2,520 c.c. and contained no sugar and only a trace of albumin.

Dog C 32.—Bull dog. The urine obtained by catheterization before the beginning of the experiment was negative for sugar and albumin. 1,200 c.c. of freshly prepared, sterile 0.9 per cent. sodium chlorid solution were introduced into the right internal jugular vein.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.
15 min.	19 c.c.	0	0
30 min.	34 c.c.	0	0
45 min.	32 c.c.	0	0
1 hr.	31 c.c.	0	0
1 hr. 15 min.	25 c.c.	0	0
1 hr. 30 min.	13 c.c.	0	0
1 hr. 45 min.	5 c.c.	0	0
2 hrs.	15 c.c.	0	0

The urine collected for twenty-four hours after the experiment amounted to 1,620 c.c. and contained no sugar or albumin.

Dog C 19.—Fox terrier, female; weight 23.5 pounds. The urine obtained by catheterization before the beginning of the experiment was negative for sugar and albumin. 2,000 c.c. of freshly prepared, sterile 0.9 per cent. sodium chlorid solution were introduced into the right internal jugular vein.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	
15 min.	75 c.c.	0	0	
30 min.	158 c.c.	0	0	
45 min.	187 c.c.	0	0	
1 hr.	138 c.c.	0	0	Introduction discontinued.
1 hr. 15 min.	130 c.c.	0	0	
1 hr. 30 min.	108 c.c.	0	0	
1 hr. 45 min.	82 c.c.	0	0	
2 hrs.	70 c.c.	0	0	

The urine collected for twenty-four hours after the experiment amounted to 780 c.c. and contained no sugar or albumin.

Dog C 43.—Fox terrier, female; weight 14.5 pounds. The urine obtained by catheterization before the beginning of the experiment was negative for sugar and albumin. 2,000 c.c. of freshly prepared, sterile 0.9 per cent. sodium chlorid solution were introduced into the left internal jugular vein.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	
15 min.	9 c.c.	0	0	
30 min.	48 c.c.	0	0	
45 min.	134 c.c.	0	0	
60 min.	131 c.c.	0	0	
1 hr. 15 min.	125 c.c.	0	0	
1 hr. 30 min.	178 c.c.	0	0	Introduction discontinued.
1 hr. 45 min.				
2 hrs.	175 c.c.	0	0	
2 hrs. 30 min.	65 c.c.	0	0	
3 hrs.	75 c.c.	0	0	
3 hrs. 30 min.	50 c.c.	0	0	

These experiments suffice to show that the introduction of physiological saline solution as described above does not cause the appearance of sugar in the urine, although in every case a marked diuresis occurred. In none of the experiments were there any definite respiratory disturbances and the animals made little resistance to the introduction of the solution. Other experiments gave concordant results, but are omitted here since their protocols show nothing of importance that the above recorded experiments do not fully demonstrate. In some of these experiments the blood sugar was studied quantitatively and the findings will be given later under another heading.

It was obvious, then, that the addition of ether to the saline solution gave to the solution the property of causing anesthesia and also the power of inducing the passage of a reducing substance into the urine.

It was then thought advisable to study more closely the mode of appearance of the glycosuria and the mechanism of its production. A series of experiments was undertaken for this purpose. In some of these a quantitative estimation was made of the sugar passed in the fractional catheterization and in the twenty-four hour voidings, and this will be found in the protocols given below. The blood sugar was also investigated and the results will be given later with similar findings in the saline series.

SERIES III. THE INTRAVENOUS ADMINISTRATION OF 5 PER CENT. ETHER IN FRESHLY PREPARED, STERILE 0.9 PER CENT. SODIUM CHLORID SOLUTION.

Dog C 20.—Fox hound; weight 34 pounds. The urine obtained by catheterization before the beginning of the experiment was negative for sugar and albumin. 1,500 c.c. of 5 per cent. ether in a freshly prepared, sterile 0.9 per cent. sodium chlorid solution were introduced.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	Sugar in gm.	
30 min.	24 c.c.	0	0	0	
45 min.	13 c.c.	0	0	0	Under surgical anesthesia.
60 min.	22 c.c.	+	0	0.0005	
1 hr. 15 min.	66 c.c.	+	0	0.1238	
1 hr. 30 min.	91 c.c.	+	0	0.1813	
1 hr. 45 min.	100 c.c.	+	0	0.1360	Introduction discontinued.
2 hrs.	50 c.c.	+	0	0.1284	
2 hrs. 15 min.	40 c.c.	+	0	0.0008	
2 hrs. 30 min.	15 c.c.	+	0	0.0005	
2 hrs. 45 min.	9 c.c.	+	0	0.0002	
3 hrs.	6 c.c.	+	0	0.0004	

The urine collected for twenty-four hours after the experiment amounted to 900 c.c. and contained 0.2160 gm. of dextrose but only a trace of albumin.

Dog C 28.—Bull dog. The urine obtained by catheterization before the be-

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	Sugar in gm.	
15 min.	32 c.c.	0	0	0	
30 min.	5 c.c.	0	0	0	
45 min.	18 c.c.	+	0	0.0035	Under surgical anesthesia.
60 min.	46 c.c.	+	0	0.0216	
1 hr. 15 min.	54 c.c.	+	0	0.0343	
1 hr. 30 min.	92 c.c.	+	0	0.0543	
1 hr. 45 min.	32 c.c.	+	0	0.0250	
2 hrs.	23 c.c.	+	0	0.0309	
2 hrs. 15 min.	17 c.c.	+	0	0.0328	
2 hrs. 30 min.	15 c.c.	+	0	0.0005	
2 hrs. 45 min.	9 c.c.	+	0	0.0008	
3 hrs.	7 c.c.	Slight	0	0.0001	

The urine collected for twenty-four hours after the experiment amounted to 1,020 c.c. and contained 1.9110 gm. of dextrose but no albumin.

ginning of the experiment was negative for sugar and albumin. 2,000 c.c. of 5 per cent. ether in a freshly prepared, sterile 0.9 per cent. sodium chlorid solution were introduced.

Dog C 35.—Bull dog, female; weight 25 pounds. The urine obtained by catheterization before the beginning of the experiment was negative for sugar and albumin. 1,600 c.c. of a solution of 5 per cent. ether in a freshly prepared, sterile 0.9 per cent. sodium chlorid solution were introduced.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	Sugar in gm.	
15 min.	11 c.c.	0	0	0	
30 min.	38 c.c.	+	0	0.0002	Under surgical anesthesia.
45 min.	42 c.c.	0	0		
60 min.	60 c.c.	+	0	0.0067	
1 hr. 15 min.	68 c.c.	+	0	0.0273	
1 hr. 30 min.	14 c.c.	+	0	0.0832	Introduction discontinued.
1 hr. 45 min.	19 c.c.	+	0		
2 hrs.	13 c.c.	+	0		
2 hrs. 15 min.	10 c.c.	+	0	0.1375	
2 hrs. 30 min.	6 c.c.	Trace	0		
2 hrs. 45 min.	4 c.c.	0	0		
3 hrs.	0	0	0		

The urine collected for twenty-four hours after the experiment amounted to 1,160 c.c. and contained 0.09280 gm. of sugar and no albumin.

Dog 36.—Wire haired mongrel, female; weight 24 pounds. The urine obtained by catheterization before the beginning of the experiment was negative for sugar and albumin. 2,000 c.c. of a solution of 5 per cent. ether in a freshly prepared, sterile 0.9 per cent. sodium chlorid solution were introduced.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	Sugar in gm.	
15 min.	10 c.c.	0	0		
30 min.	32 c.c.	0	0		
45 min.	61 c.c.	0	0		
60 min.	41 c.c.	+	0	0.0026	Under surgical anesthesia.
1 hr. 15 min.	83 c.c.	+	0	0.0053	
1 hr. 30 min.	102 c.c.	+	0	0.0065	
1 hr. 45 min.	93 c.c.	+	0	0.0059	Introduction discontinued.
2 hrs.	40 c.c.	+	0	0.0060	
2 hrs. 15 min.	112 c.c.	+	0	0.0077	

The urine collected for twenty-four hours after the experiment amounted to 2,200 c.c. and contained 0.1776 gm. of sugar and no albumin.

Dog C 40.—Mongrel, female; weight 16 pounds. The urine obtained by catheterization before the beginning of the experiment was negative for sugar and albumin. A 5 per cent. solution of ether in a freshly prepared, sterile 0.9 per cent. sodium chlorid solution was introduced.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	Sugar in gm.	
15 min.	21 c.c.	0	0	0	
30 min.	22 c.c.	+	0	0	
45 min.	50 c.c.	+	0	0.0734	Under surgical anesthesia.
60 min.	71 c.c.	+	0	0.0676	
1 hr. 15 min.	42 c.c.	+	0	0.1312	
1 hr. 30 min.	41 c.c.	+	0	0.0882	Introduction discontinued.
1 hr. 45 min.	11 c.c.	+	0	0.1167	
2 hrs.	8 c.c.	+	0		
2 hrs. 15 min.	0	0	0		
2 hrs. 30 min.	0	0	0		

The urine collected for twenty-four hours after the experiment amounted to 500 c.c. and contained 0.1096 gm. of sugar but no albumin.

Dog C 43.—Fox terrier, female; weight 12 pounds. The urine obtained by catheterization before the beginning of the experiment was negative for sugar and albumin. 1,200 c.c. of a solution of 5 per cent. ether in a freshly prepared sterile 0.9 per cent. sodium chlorid solution were introduced into the right jugular vein.

Time.	Amount of urine.	Sugar in urine.	Albumin in urine.	
15 min.	6 c.c.	0	0	
30 min.	5 c.c.	0	0	
45 min.	8 c.c.	+	0	
60 min.	11 c.c.	+	0	Under surgical anesthesia.
1 hr. 15 min.	14 c.c.	+	0	
1 hr. 45 min.	68 c.c.	+	0	Introduction discontinued.
2 hrs.	82 c.c.	+	0	
2 hrs. 15 min.	40 c.c.	−	0	
2 hrs. 45 min.	58 c.c.	−	0	

The urine collected for twenty-four hours after the experiment amounted to 1,190 c.c. and contained some sugar but no albumin.

This series verifies the findings in our preliminary experiment. A closer study of the results brings out the following points.

The Flow of Urine.—Hawk (13) has found that ether administered by the inhalation method inhibits the flow of urine during the anesthesia, but after the anesthesia is over the flow is accelerated.

In our experiments it was observed in general that the amount of urine voided during the fifteen minute intervals was considerably diminished in the ether-saline transfusion as compared with the saline transfusions alone, as is shown by the next table.

In practically every case the total amounts of urine voided during the experiments and for twenty-four hours afterwards were less in

Time.	Dog *C* 43. Saline 0.9 % alone (1,200 c.c.).	Dog *C* 43. Saline 0.9 % and ether 5 % (1,200 c.c.).	Dog *C* 28. Saline 0.9 % alone (2,000 c.c.).	Dog *C* 28. Saline 0.9 % and ether 5 % (2,000 c.c.).
15 min.	9 c.c.	6 c.c.	3 c.c.	32 c.c. (?)
30 min.	48 c.c.	5 c.c.	13 c.c.	5 c.c.
45 min.	134 c.c.	8 c.c.	35 c.c.	18 c.c.
60 min.	131 c.c.	11 c.c.	62 c.c.	46 c.c.
1 hr. 15 min.	125 c.c.	14 c.c.	75 c.c.	54 c.c.
1 hr. 30 min.			55 c.c.	92 c.c.
1 hr. 45 min.	178 c.c.	68 c.c.	50 c.c.	32 c.c.
2 hrs.	175 c.c.	82 c.c.	60 c.c.	23 c.c.
2 hrs. 15 min.		40 c.c.	48 c.c.	17 c.c.
2 hrs. 30 min.	65 c.c.		110 c.c.	15 c.c.
2 hrs. 45 min.		58 c.c.	58 c.c.	9 c.c.

the saline-ether than in the saline tranfusion alone. The comparative voidings contrast strikingly and this is shown in the table given below.

Saline transfusion.

Dog.	Amount of solution introduced.	Amount of urine during experiment.	Amount of urine for 24 hours.	Diuresis.
C 20	1,700 c.c.	719 c.c.	2,500 c.c.	1,519 c.c.
C 28	2,000 c.c.	799 c.c.	2,520 c.c.	1,319 c.c.
C 43	2,000 c.c.	990 c.c.	1,800 c.c.	790 c.c.
C 20	1,800 c.c.	634 c.c.	3,100 c.c.	1,934 c.c.

Saline and ether transfusion.

Dog.	Amount of solution introduced.	Amount of urine during experiment.	Amount of urine for 24 hours.	Diuresis.
C 20	1,500 c.c.	350 c.c.	1,020 c.c.	130 c.c.
C 28	2,000 c.c.	350 c.c.	1,020 c.c.	630 c.c.
C 43	1,600 c.c.	292 c.c.	1,190 c.c.	118 c.c.
C 20	1,500 c.c.	436 c.c.	900 c.c.	164 c.c.

Evidently the addition of ether to the saline solution modifies markedly the extent of filtration through the kidney. The striking diuresis which follows the saline transfusion gives place to an actual retention of fluid as measured by the comparison of the total output with the intake. The amount of fluid lost through evaporation from the lungs and by the saliva necessarily is of importance in judging the relation of the fluid output to the intake. But for our purpose the results given above indicate with sufficient accuracy that ether exerts an inhibiting effect upon filtration through the kidney.

Furthermore, it has been reported by Osterhaut (14) that the penetration of living cells by ions is retarded by anesthetics.

The Glycosuria.—It is of interest to find that the reducing body does not appear in the urine until after anesthesia has been produced. Absence of reflexes, flaccidity of the extremities, and marked dilatation of the pupils,—indicating the condition of anesthesia,—were always present to a varying degree prior to the appearance of glycosuria. The phenomenon of glycosuria in this type is very intimately related to the state of the anesthesia.

In the majority of our experiments the reducing body was present in the urine as long as fifteen minute catheterizations showed that there was an appreciable flow. On the average, this flow lasted about one hour and forty-five minutes after the introduction of the saline had been discontinued. There was always a varying amount of sugar in the urine collected for twenty-four hours. The extent of the glycosuria with relation to the amount of fluid transfused is shown in the table that follows.

Dog.	Amount of fluid transfused.	Amount of ether in fluid.	Dextrose in gm. voided during experiment.	Dextrose in gm. in 24 hrs.	Total amount of dextrose in gm.	Amount of 24 hr. urine.
C 20	1,500 c.c.	75 c.c.	0.6620	0.2160	0.8780	900 c.c.
C 28	2,000 c.c.	100 c.c.	0.2038	1.9110	2.1148	1,020 c.c.
C 21	1,800 c.c.	90 c.c.	0.0148	1.4120	1.4268	1,200 c.c.
C 35	1,600 c.c.	80 c.c.	0.2549	0.0928	0.3477	1,160 c.c.
C 36	2,000 c.c.	100 c.c.	0.0340	0.1776	0.2116	2,200 c.c.
C 40	1,600 c.c.	80 c.c.	0.4771	0.1096	0.5867	500 c.c.

In a general way the extent of the glycosuria is proportional to the amount of ether introduced into the veins, and consequently to the depth of the anesthesia. A similar relationship was found for the glycosuria following the respiratory method of anesthesia.

The Change in the Percentage of Dextrose in the Blood.—It would be of great importance to know what change in the percentage of dextrose in the blood, if any, was associated with this type of glycosuria. Accordingly, by the method described in a previous communication, samples of blood were taken before the beginning of the experiment and after the discontinuance of the transfusions. These were analyzed for their percentage of dextrose by the Oppler-Bertrand (7) method.

The first point to be investigated was the percentage of dextrose in the blood before and after the simple saline transfusions, which

were not associated with glycosuria or anesthesia. The results are tabulated below.

Dog.	Per cent. of dextrose in blood before transfusion.	Per cent. of dextrose in blood after transfusion.	Change in percentage of dextrose in per cent.	Remarks.
C 21	0.074	0.077	+0.003	Very slight change.
C 28	0.078	0.094	+0.016	Very slight change.
C 36	0.041	0.050	+0.009	

Following simple saline (0.9 per cent.) transfusion, there is, then, practically no change in the percentage of dextrose in the blood before and after the transfusion.

With the effect of the saline component of the solution upon the sugar in the blood thus settled, the study of the change in percentage of dextrose in the blood following the saline-ether transfusion was undertaken. The results are given in tabular form.

Dog.	Per cent. of dextrose in blood before transfusion.	Per cent. of dextrose in blood after transfusion.	Increase in dextrose in per cent.
C 40	0.040	0.105	0.065
C 30	0.040	0.109	0.069
C 38	0.040	0.214	0.174
C 36	0.044	0.135	0.090
C 28	0.049	0.071	0.022
C 43	0.053	0.089	0.035

In some of the experiments the percentage of dextrose was so strikingly increased as a result of the saline-ether transfusion that it exceeded the upper limits of normal and denoted a true hyperglycemia. In other experiments there was only a slight change. In every case, however, the change was greater than that following the pure saline transfusions.

Thus it appears that the glycosuria in this type of anesthesia is more closely allied to a true hyperglycemia.

The rectal temperature was followed during the course of some of the experiments and may be just touched upon here. Time did

Dog *C* 32.		Saline and ether transfusion.						
Time.	Before.	15 min.	45 min.	1 hr. 15 min.	1 hr. 45 min.	2 hrs. 15 min.	2 hrs. 30 min.	2 hrs. 45 min.
Temperature C.	38.6°	37.0°	36.°	35.5° Transfusion discontinued.	38.°	38.7°	38.8°	39.9°

not permit a detailed investigation, and so no discussion of this feature of the transfusion will be attempted.

Dog *C* 43.	Before.	Saline and ether transfusion.				
Time.		30 min.	1 hr. 15 min.	2 hrs. 15 min.	2 hrs. 45 min.	22 hrs.
Temperature C.	38.65°	36.1°	36.8° Transfusion discontinued.	39.8°	39.6°	39.4°

Dog *C* 43.	Before.	Saline transfusion.									
Time.		15 min.	30 min.	1 hr.	1 hr. 15 min.	1 hr. 30 min.	2 hrs.	2 hrs. 30 min.	3 hrs.	3 hrs. 30 min.	22 hrs.
Temperature C.	39.5°[1]	39.2°	39.1°	40.3°	40.3°	40.8°	40.5°	40.5°	39.9°	39.9°	39.8°

In all the experiments tabulated a rise of temperature is seen.

DISCUSSION.

The intravenous method of anesthesia as outlined in the preceding experiments produces a very satisfactory surgical anesthesia without any demonstrable signs of asphyxia. It is difficult to conceive that asphyxia plays a rôle here in the causation of the glycosuria, since blood taken at the end of the transfusion, while the animal was still deeply anesthetized, was never darkly venous in any of the experiments. The mucous membranes gave no evidences of cyanosis and the animals were in all cases breathing the air of the room.

In some of the experiments there was considerable preliminary excitement during the introduction of the ether-saline solution. Before anesthesia was produced, struggling and rapid respirations were often observed, but were not followed by any noticeable periods of apnea. The respirations during the anesthesia were remarkably even in rhythm and depth. Whether acapnia is very intimately concerned as a causative factor in this type of glycosuria cannot, of course, be stated with certainty until analyses of the gases of the blood have been made. But no such analyses are available for this

[1] The temperature was still elevated from the result of the saline-ether transfusion.

paper. However, the cycle of hyperpnea followed by apnea, which has been especially emphasized by Henderson (3), was not noticeably evident in any of our studies.

Apparently the ether must reach a concentration in the blood sufficient to produce anesthesia before glycosuria occurs. Blood taken at the end of the transfusion is always rich in ether. During the course of the transfusion ether is eliminated rapidly by the lungs. Occurring simultaneously with this there is always a greatly increased flow of saliva.

The question of whether the anesthetic administered in this way causes a diminished oxidation on the part of the tissues must be considered. Here again no exact analyses of the oxygen percentages in the blood in our experiments are available at this time. However, asphyxia, which is one of the causative factors in diminished oxidation, has been largely prevented. In spite of this, however, glycosuria occurs.

Subsequently other studies will be presented which are directed more especially to the gaseous metabolism during intravenous anesthesia.

The main features regarding the production of glycosuria by the respiratory and intravenous routes agree quite strikingly. In the former series of studies the hyperglycemia and glycosuria were on the average quantitatively greater. This is in all probability due to the larger amount of ether used in that series.

CONCLUSIONS.

1. By introducing intravenously into dogs a solution of 5 per cent. ether in normal saline (0.9 per cent.), a satisfactory surgical anesthesia can be produced.

2. Associated with this anesthesia a glycosuria occurs in healthy adult dogs fed on a meat diet.

3. Normal saline (0.9 per cent.) introduced in exactly the same manner does not cause a glycosuria.

4. The glycosuria is, in general, associated with a hyperglycemia.

5. The glycosuria does not make its appearance until the condition of anesthesia has been produced.

6. The intravenous method of producing anesthesia employed in our studies eliminates largely the factor of asphyxia.

7. Consequently by this mode of administration ether may cause glycosuria without asphyxia.

8. The addition of ether to normal saline (0.9 per cent.) solution modifies the property of the latter to produce diuresis.

9. Associated with the intravenous administration of both the saline and the ether-saline solutions there occurs a rise of body temperature.

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TREPONEMA MUCOSUM (NEW SPECIES), A MUCIN-PRODUCING SPIROCHÆTA FROM PYORRHEA ALVEOLARIS, GROWN IN PURE CULTURE.*

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PLATE 18.

Pyorrhea alveolaris is generally regarded as the sequel to a pathological metabolism known as the uric acid diathesis. Whatever the primary predisposing factors may be, the characteristic fetid odor from the affected mouth betrays the presence of certain putrefactive microörganisms in the perialveolar tissues which surround the loosened roots of the diseased teeth. The bacteriological studies which, on account of the lack of cultural methods for most of the organisms concerned, were necessarily limited to their morphology, helped but little, for the varieties of the microbes therein found do not differ greatly morphologically from those commonly seen in the gingival deposit on unclean teeth of individuals who do not have pyorrhea alveolaris. Most authorities mention the presence of *Spirochæta buccalis, Treponema macrodentium, Treponema microdentium,* fusiform bacilli, and, occasionally, a spirillum allied to that of Vincent. But which of these organisms is most responsible for the fetid odor could not be determined for the reason that until very recently neither the spirochætæ nor the other forms were isolated in pure cultures.

The fusiform bacilli and spirilla of Vincent's type have been obtained in pure culture by Ellermann,[1] Weaver and Tunnicliff,[2] Mühlens,[3] and others,[4] who found that these organisms produce the peculiar fetid odor characteristic of pyorrhea alveolaris.

* Received for publication, May 15, 1912.

[1] Ellermann, V., *Centralbl. f. Bacteriol., Orig.*, 1904, xxxvii, 729.

[2] Weaver, G. H., and Tunnicliff, R., *Jour. Infect. Dis.*, 1905, ii, 446.

[3] Mühlens, P., *Deutsch. med. Wchnschr.*, 1906, xxxii, 797.

[4] Tunnicliff, R., *Jour. Infect. Dis.*, 1906, iii, 148.

Of the spirochætæ, one medium type, *Treponema macrodentium,* and one small type, *Treponema microdentium,* have been cultivated by me from non-pyorrheal cases,[5] but the macrodentium produces no odor and the microdentium produces a putrefactive odor which is somewhat different in nature from that which comes from pyorrhea alveolaris.

In the present communication I shall describe a small spirochæta which was isolated from the pus derived from a case of pyorrhea alveolaris. Morphologically the organism resembles greatly *Treponema pallidum* and *Treponema microdentium,* but it differs from both in several principal biological properties. Its most striking feature is its capacity to produce in pure culture a mucin and a strong fetid odor. Since this species has, for the first time, been recognized and isolated from other varieties, I propose for it the name *Treponema mucosum.* At present I shall not discuss the question of how much this particular spirochæta contributes towards the production of the fetid odor in the pyorrheal discharge, but I shall describe the method of cultivation and its morphology and biology in pure culture.

Method of Cultivation.—The purulent discharge of pyorrheal teeth is collected by means of a sterile capillary pipette and then suspended in a few cubic centimeters of sterile citrate solution. By means of a capillary pipette this emulsion is inoculated into a number of tubes of solid culture media consisting of one part of ascitic fluid, two parts of ordinary agar, and having a piece of sterile fresh rabbit kidney at the bottom; the whole medium is covered with a layer of sterile paraffin oil. The technique is the same as that used for obtaining the pallidum or refringens from a contaminated chancre or condyloma. After inoculation the tubes are incubated at 37° C. for about ten days. The whitish semi-transparent growth may be seen to spread out from the stab canal which is now filled with a dense growth of various bacteria. The tube is cut open at the middle, the freshly exposed surface of the agar column is carefully sterilized with a sublimate alcohol solution, the moisture carefully wiped off and then the cloudy colony of the spirochætæ is reached by inserting a sterile capillary pipette through

[5] Noguchi, H., *Jour. Exper. Med.,* 1912, xv, 81.

the clean surface. A small portion of the colony is thus taken out and transferred to a new medium. By repeating this process of purification one finally obtains a pure culture.

Properties of the Pure Cultures.—In the ascitic agar medium, containing fresh sterile tissue, the colonies become visible within twenty-four to forty-eight hours, and with the exception of the upper 1.5 centimeters the entire agar column is rendered unevenly opalescent. The individual colonies are recognized as dense, whitish turbidities of varying sizes concentrically arranged but having no definite outer boundaries. In a rapidly growing culture the neighboring colonies unite with each other by their gradually thinning peripheries, thus making the whole tube quite opalescent, although the original colonies still stand out more or less distinctly.

No gas is formed. The culture gives off a strong fetid odor that is easily recognized at a distance. After growing for two or three weeks the tissue in the culture becomes at first greyish and then quite dark (similar changes were found in the microdentium culture). The culture now contains a varying amount of mucin. When the media is broken up this is seen as an extremely fine string stretching from one piece of agar to another. The mucin string is not sticky, but draws out as a fine filament. I have seen no bacteria which produce a similar mucin.

The organisms remain alive for several weeks in the same culture at 37° C. The mucin-producing faculty has apparently suffered through repeated transplantations and at present, in its sixteenth generation within 160 days, the culture has almost lost this quality. On the other hand, its capacity to produce a bad odor has in no wise diminished.

Like other treponemata, the mucosum is an anaerobe and requires the presence of serum constituents for its growth. It grows well without the presence of fresh tissue. The fluid medium is made markedly opalescent without forming a definite coagulum. In this fluid medium the growth produces a very strong odor.

Morphologically *Treponema mucosum* resembles both the pallidum and the microdentium, measuring on the average 8 to 12 microns by 0.25 to 0.3 of a micron (figures 1 and 2, *Treponema mucosum;* figures 3 and 4, *Treponema microdentium;* figures 5 and

6, *Treponema pallidum*). The number of curves varies from six to eight. These curves are remarkably regular and are often quite deep. Both extremities are sharply pointed and often possess at one or both ends a fine, minutely curved projection that varies in length in different organisms. The length of this projection may reach eight to ten microns. The organisms show a graceful rotating movement, and are often seen joined to each other by a thin filament forming a pair, or chain of three, four, or more individuals. Under certain cultural conditions forms suggestive of a longitudinal division are observed.

When cultivated under unfavorable conditions a large number of irregular forms appear. Some of these are almost straight and some are slightly or irregularly curved. There are also many granular particles. These particles may be merely degenerative products or they may be segments which under favorable conditions are capable of reproducing the spirochætæ. These segments or granules take the chromatin stain and vary in size. Not infrequently a long spirochæta is found undergoing a granular segmentation (degeneration?), or a small spirochæta is seen attached to a round body as if it had just sprouted out of the latter. In its staining reactions the mucosum behaves as the microdentium does and takes the red in the Giemsa stain.

Pathogenicity.—When a large quantity of active fluid culture is inoculated into the cutaneous tissue of the skin of a *Macacus rhesus* monkey, it produces an acute inflammation and the tissues remain indurated for one week or ten days. There is no tendency to suppuration, and after twenty-four hours the tissue does not contain living spirochætæ. In the testicular tissue of rabbits the inoculated organisms produce also a marked induration (acute inflammatory) which may last for one week, but no abscess formation was observed. The testicle returns to its normal appearance in about two weeks.

When a culture in ascitic agar (as an emulsion) is inoculated, the testicle becomes intensely inflamed within twenty-four hours and remains so for nearly ten days. The inflammation then gradually subsides, but the hard circumscribed nodule persists for many weeks. When the testicle is punctured with a capillary pipette a yellowish thick pus flows out. This pus has a fetid odor and in it a small

number of the spirochætæ are still to be seen. By transplanting the pus into a new culture medium a pure growth of this organism can be obtained. From this fact it seems that the mucosum can survive in the testicle of rabbits when there is simultaneously introduced a foreign substance (agar), but not without the aid of the latter; hence it is not an independent parasite.

Differentiation from the Allied Species.—The mucosum differs from the microdentium in producing a mucin, a stronger fetid odor, a denser growth, and in surviving in the rabbit testicle when introduced with agar. Morphologically it is almost impossible to differentiate these two species. The pallidum differs from this organism by its pathogenicity, by producing no odor or mucin, by its fainter diffuse growth, and by requiring for its growth the presence of fresh tissue in the media.

CONCLUSIONS.

1. A mucin-producing spirochæta has been obtained in pure culture from a case of pyorrhea alveolaris. This organism is an independent species of the genus Treponema and, as it is recognized for the first time, I propose for it the name *Treponema mucosum.*

2. Morphologically the mucosum is difficult to separate from the pallidum and microdentium, but, through its biological properties and animal reactions it is easily differentiated from all the rest of the spirochætæ.

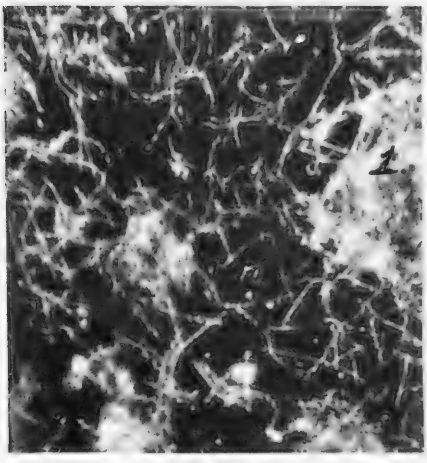
3. The mucosum is not parasitic in the strict sense of the term, but exerts a certain pyogenous action when the tissue has been so injured by foreign substances as to enable it to survive.

4. The strong fetid odor in the discharge from pyorrhea alveolaris is due, at least in part, to the presence of the mucosum in the affected tissue.

EXPLANATION OF PLATE 18.

Dark-field views of the organisms. × 1,100.

FIGS. 1 and 2. *Treponema mucosum* from pure cultures ten days old.
FIGS. 3 and 4. *Treponema microdentium* from pure cultures ten days old.
FIGS. 5 and 6. *Treponema pallidum* from pure cultures two weeks old.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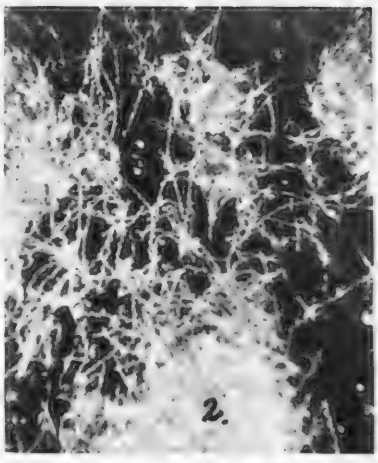
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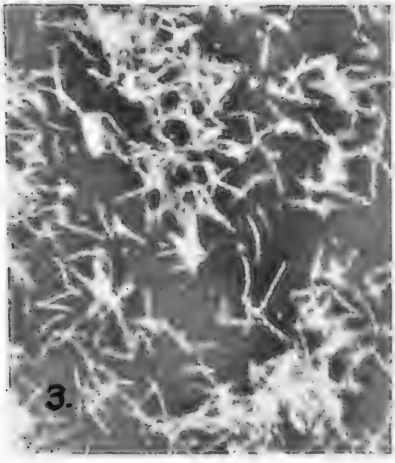
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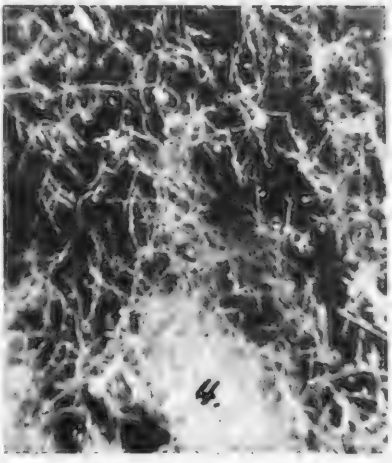
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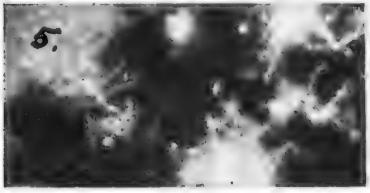
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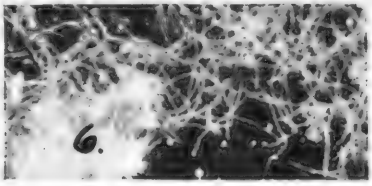
6.

THE PURE CULTIVATION OF SPIROCHÆTA DUTTONI, SPIROCHÆTA KOCHI, SPIROCHÆTA OBERMEIERI, AND SPIROCHÆTA NOVYI.*

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PLATES 19 AND 20.

There are at least four distinct species of spirochætæ that are responsible for the diseases known as relapsing fever. The organisms in the blood of patients suffering from the relapsing fever of Europe were first discovered by Obermeier[1] in 1873, and since then this species has been known as *Spirochæta obermeieri.* The second variety is found in the blood of those who have contracted the disease known as African tick fever; it was discovered in 1904 simultaneously by Ross and Milne,[2] and by Dutton and Todd.[3] This variety bears the name of *Spirochæta duttoni.* In 1905 the third species was discovered in Africa by Koch[4] who assumed that it was identical with other varieties already known at that time; but the recent investigations of Novy and Fraenkel[5] indicate that Koch's organism is distinct from the obermeieri or the duttoni. For Koch's organism Novy proposed the name *Spirochæta kochi.* The fourth species, *Spirochæta novyi,* was found by Norris[6] in 1906 in New York in the blood of a patient with relapsing fever, and was afterwards transmitted to rats and mice. Although very similar in morphology to *Spirochæta obermeieri,* Fraenkel,[7] Uhlenhuth and

* Received for publication, June 1, 1912.

[1] Obermeier, O., *Berl. klin. Wchnschr.,* 1873, x, 152, 378, 391, 455.

[2] Ross, P. H., and Milne, A. D., *Brit. Med. Jour.,* 1904, ii, 1453.

[3] Dutton, J. E., and Todd, J. L., *Brit. Med. Jour.,* 1905, ii, 1259.

[4] Koch, R., *Deutsch. med. Wchnschr.,* 1905, xxxi, 1865; *Berl. klin. Wchnschr.,* 1906, xliii, 185.

[5] Fraenkel, C., *Med. Klin.,* 1907, iii, 928; *München. med. Wchnschr.,* 1907, liv, 201; *Berl. klin. Wchnschr.,* 1907, xliv, 681; *Hyg. Rundschau,* 1907, xvii, 263.

[6] Norris, C., Pappenheimer, A. M., and Flournoy, T., *Jour. Infect. Dis.,* 1906, iii, 266.

[7] Fraenkel, C., *Berl. klin. Wchnschr., loc. cit.*

Haendel,[8] Schellack,[9] Strong,[10] Manteufel,[11] and others, consider *Spirochæta novyi* to be a distinct species, chiefly on account of the immunity phenomena.[12]

There is another variety which was described by Carter[13] in India, but it is not yet definitely determined whether or not this is identical with *Spirochæta novyi* or *Spirochæta obermeieri,* although Novy considers it a distinct species on account of its being somewhat thinner than any of the other organisms.

Schellack gives the following comparison of various species:

Spirochæta duttoni: length, 24 to 30 microns; width, 0.45 of a micron.
Spirochæta obermeieri: length, 19 to 20 microns; width, 0.39 of a micron.
Spirochæta novyi: length, 17 to 20 microns; width, 0.31 of a micron.

My success in causing other spirochætæ to grow in culture media made it seem advisable for me to apply similar principles to the blood spirochætæ, *Spirochæta duttoni, Spirochæta kochi, Spirochæta obermeieri,* and *Spirochæta novyi,* especially as no one had succeeded in obtaining *in vitro* a culture of any of these spirochætæ in which growth was kept up for many generations by transplanting the organisms from culture to culture.[14]

There are some investigators who observed a decided increase in the number of the organisms when citrated blood was kept at room temperature or in the thermostat, but they failed to obtain a second generation. According to Novy and Knapp, blood containing the spirochætæ may remain infectious for many days, but no true cultivation is effected.

[8] Uhlenhuth, P., and Haendel, *Arb. a. d. k. Gsndhtsamte.,* 1907, xxvi, 1.
[9] Schellack, C., *Arb. a. d. k. Gsndhtsamte.,* 1908, xxvii, 364.
[10] Strong, R. P., *Philippine Jour. Sc.,* 1908, iii, 231.
[11] Manteufel, *Arb. a. d. k. Gsndhtsamte.,* 1908, xxvii, 327.
[12] Novy, F. G., and Knapp, R. E., *Jour. Infect. Dis.,* 1906, iii, 291. Breinl, A., and Kinghorn, A., *Lancet,* 1906, i, 668; *Mem. Liverpool School Trop. Med.,* 1906, xx, 61.
[13] Carter, H. V., *Deutsch. med. Wchnschr.,* 1879, v, 189, 351, 386.
[14] Williams, L. A. and R. S., *Mem. Liverpool School Trop. Med.,* 1906, xxi, 101. Duval, C. M., and Todd, J. L., *Lancet,* 1909, i, 834. Levaditi, C. (*Compt. rend. Acad. d. sc.,* 1906, cxlii, 1099), and Novy, F. G., and Knapp, R. E. (*Jour. Am. Med. Assn.,* 1906, xlvii, 2152), succeeded in keeping up the growth of *Spirochæta kochi* and *Spirochæta novyi* (formerly thought to be the obermeieri by Novy), respectively, for many generations by means of collodion sacs placed in the peritoneal cavity of rabbits; but their work does not enter into consideration in the discussion of cultivation *in vitro.*

As will be described in detail later, I have found the conditions that are necessary for obtaining cultures *in vitro* of the organisms of the relapsing fevers. These conditions are somewhat different from those required by the other spirochætæ I have cultivated. Thus, for the pallidum, microdentium, macrodentium, mucosum, refringens, and phagedenis, a strict anaerobiosis is necessary; while for the relapsing fever spirochætæ the presence of oxygen seems to be essential. My cultivation experiments will be reported in the following pages.

Material for Cultivation.[15]—Four different strains of spirochætæ were employed for the experiments here described: *Spirochæta duttoni, Spirochæta kochi, Spirochæta obermeieri,* and *Spirochæta novyi.* All of these have been preserved for many years in rats or mice.

Method of Cultivation.—After trying various culture media and methods I found the following to be the most suitable and reliable for the growth of all of the blood spirochætæ. Into each of a number of sterile test-tubes (I use tubes 2 by 20 centimeters) is placed a piece (not too small) of sterile fresh tissue, usually rabbit kidney, and then are added a few drops of citrated blood from the heart of the infected mouse or rat. Following this, about fifteen cubic centimeters of sterile ascitic or hydrocele fluid are quickly poured into the tubes, and the contents of some of the tubes are covered with a layer of sterile paraffin oil, while the rest are left without the oil. The tubes are now transferred to a thermostat at 37° C.

It is essential to obtain the blood from the infected animal absolutely aseptically. In my experiments, under ether anesthesia, the blood was drawn directly from the heart and was then mixed immediately with citrate solution (1.5 per cent. sodium citrate in physiological saline solution). It is best to obtain the blood between the forty-eighth and the seventy-second hours after the inoculation of the animal. The blood suspension thus prepared was examined for the spirochætæ under the dark-field microscope and was then used for the inoculation of the culture tubes.

[15] I am under great obligation to Professor F. G. Novy for placing at my disposal his strains of *Spirochæta duttoni, Spirochæta obermeieri,* and *Spirochæta novyi.* The *Spirochæta kochi* employed by me was obtained from Dr. B. T. Terry.

It is very important to employ samples of ascitic fluids which contain no bile, but which form a loose fibrin in the culture tube, for many specimens are unsuitable just because they contain too much bile or do not cause the formation of fibrin when mixed with the fresh tissue in the culture tube. Fluids which have been heated to 56° to 60° C. for thirty minutes, or which have been filtered through the Berkefeld filter are unsuitable. The addition of bouillon or sugar to the fluids also diminishes their cultural value. Sterile tissue kept a day or two on ice is less suitable for cultivation purposes than fresh tissue, and may prove not at all suitable.

It is not necessary to introduce a large number of spirochætæ into the culture tubes. An examination of the culture made immediately after my inoculations usually showed one organism in every twenty or thirty fields, but sometimes it was almost impossible to find a spirochæta even after a long search.

Results of Cultivation.—By the method described above I have succeeded in obtaining pure cultures of the four varieties with which I experimented; *i. e., Spirochæta duttoni, Spirochæta kochi, Spirochæta obermeieri,* and *Spirochæta novyi. Spirochæta kochi* was the first to be cultivated and has already gone through twenty-nine generations within six months. *Spirochæta novyi* was the last to be cultivated and is now in its fourth passage. It may be stated that subcultures can be obtained in the tissue ascitic fluid media by inoculating into it about 0.5 of a cubic centimeter of the original culture. For growth in these subcultures the addition of a small amount of normal rat or human blood is advantageous but not essential.

PURE CULTIVATION OF SPIROCHÆTA DUTTONI.

Rate of Multiplication.—When a small number of organisms is inoculated, the original culture tube shows almost no sign of multiplication within twenty-four hours. On microscopical examination, after forty-eight hours one perceives a slight increase in the number of organisms, but at the seventy-second hour the number of spirochætæ has increased decidedly. Four to six days after inoculation the organisms have increased so much that one may be seen in each field. On the eighth or ninth day the maximum in multipli-

cation is reached, and in every field many actively motile organisms may be seen singly, in chains, or in masses. On the tenth day there comes a sudden change. Not only have the spirochætæ ceased to increase, but a beginning diminution in their number may be observed. The active organisms are very few and the other spirochætæ seem to be on the verge of disintegration. The spirochætæ are seen to lose their protoplasm, and the spiral framework of the body is left bare. Many spherical bodies and irregular protoplasmic masses make their appearance. In cultures which are not covered with oil and which are more than fifteen days old it is exceptional to find an active spirochæta, but I should mention that the spirochætæ multiply somewhat more slowly and disappear also somewhat later in the tubes in which the contents are covered with paraffin oil than in the tubes containing no oil.

At 15° C. no multiplication takes place and the organisms undergo degeneration very promptly. No growth is obtainable in an atmosphere of hydrogen or *in vacuo*.

Effect upon the Culture Media.—Apparently there is no visible alteration in the culture media in which the spirochætæ are rapidly multiplying. The tissue may look somewhat paler, but no coagulation of the fluid (ascitic or hydrocele) takes place, and no odor is produced by the growth.

Transplantations.—Subcultures can be made at any time between the fourth and ninth days. It is not advisable to make the subinoculations earlier than the fourth day or after the culture has passed its period of maximum growth. By using about 0.5 to 1 cubic centimeter of the culture any number of successive transfers (so called passages) may be obtained. It is extremely important that no bacterial contamination should occur, because the simultaneous growth of certain cocci or bacilli causes the spirochætæ to disappear from the culture.

Morphology.—(Figures 1 to 5.) In young cultures short forms with only two or three curves are very numerous. In the cultures approaching the maximum growth (eighth to ninth day) the length of the organisms is about that seen in normal infections. The curves are very regular and deep. The spirochætæ may be single, paired. or in chains. They are vigorously motile. They usually show a

long, regularly curved, delicate projection at each end of the body. In old cultures, after ten days, the spirochætæ lose their motility and the protoplasmic masses concentrate at varying points along the spiral skeleton. There are many organisms which show one or more spore-like spherical bodies attached laterally at irregular intervals (figure 2). In such old cultures there are usually many spiral skeletons entangled or embedded in granular protoplasmic remains (figure 5). But even in these old cultures a few active spirochætæ are occasionally met with. Old cultures may be temporarily rejuvenated, *i. e.,* for three or four days, by introducing into them fresh ascitic fluid and a piece of sterile fresh tissue, but degeneration that is almost complete soon follows.

It is interesting to notice that the spirochætæ grown under a layer of paraffin oil are somewhat thinner than those grown in the absence of oil.

Mode of Division.—(Figure 4.) In luxuriantly growing cultures it is difficult to decide whether the spirochætæ undergo longitudinal or transverse division, as they are usually seen in pairs or chains, united to each other by a thin filament. Their general appearance suggests transverse fission, but no undoubted instance of transverse division has thus far been observed. At the same time there are always a few instances of unmistakable longitudinal division. Moreover, I have repeatedly found cultures in which, probably due to certain unfavorable conditions, almost every organism showed partial longitudinal division. When these organisms were watched for some time under the dark-field microscope, the process of longitudinal division could be observed.

Pathogenicity.—*Spirochæta duttoni* cultivated *in vitro* remains infectious for rats and mice. I have been able to infect rats and mice with cultures that were in their ninth passage, and as far as the severity of the infection is concerned no difference has been seen between that caused by cultures and that caused by inoculating blood obtained from infected animals. But, as will be pointed out when *Spirochæta kochi* is discussed, it is possible that a diminution in the virulence may yet be observed when the number of passages has become sufficiently large.

PURE CULTIVATION OF SPIROCHÆTA KOCHI.

Spirochæta kochi is the first variety of the spirochætæ of relapsing fever that was successfully cultivated by me. In most respects it is very similar to *Spirochæta duttoni*. *Spirochæta kochi* has been growing *in vitro* since February, 1912, and by renewing the cultures regularly every week no difficulty has thus far been experienced in keeping the culture growing. As a rule, the maximum growth is reached on the ninth day. As in the case of *Spirochæta duttoni*, no visible alteration of the media results from the growth and there is no perceptible odor at any period of its cultivation. Morphologically (figures 6 to 11) it is almost indistinguishable from *Spirochæta duttoni* except that it is possibly a trifle thinner than the latter. It divides longitudinally and possibly also transversely.

Pathogenicity.—*Spirochæta kochi* cultivated *in vitro* retained for several passages all its usual virulence and killed rats and mice fairly constantly, but when tested after the tenth passage *in vitro* it no longer killed these animals. In the rats and mice that had been inoculated, spirochætæ developed in large numbers, but their appearance was delayed to the third or fourth day. The sixteenth passage also caused an infection in these animals, but was apparently less virulent than the tenth passage. From this it appears that the virulence of these organisms is being gradually attenuated by prolonged cultivation *in vitro*. The fourth passage of the culture failed to infect rabbits, guinea pigs, cats, dogs, a chicken, or a *Macacus rhesus* monkey.

PURE CULTIVATION OF SPIROCHÆTA OBERMEIERI.[16]

Spirochæta obermeieri multiplies more rapidly in cultures *in vitro* than *Spirochæta duttoni* or *Spirochæta kochi*. Within twenty-four hours there is a decided increase in the number of organisms in the culture and the maximum growth is usually reached on the seventh day, when every field shows several spirochætæ. The decline commences after the seventh day, and within the next two days degeneration sets in. Under the cover of paraffin oil the organisms grow more slowly and remain active for a day or two longer than in the tubes containing no oil.

[16] Synonymous with *Spirochæta recurrentis*.

Transplantations.—Successful subcultures are best obtained by inoculating new media with about one cubic centimeter of a culture that has shown active growth for three to four days, although a culture at the height of its growth (seventh day) can also be used.[17] I have found it always advantageous to provide two series of tubes, —one with and the other without the paraffin oil layer, because not infrequently the growth is more abundant and vigorous in one than in the other of these two series. I have been unable to determine the cause of this irregularity, but there is no difficulty in continuing the cultivation if both conditions are provided. No growth takes place at room temperature.

Morphology.—(Figures 12 to 17.) In young cultures (not older than three or four days) there are, besides the active specimens of average length and width, numerous very motile short organisms which show only two or three curves. Among these forms one frequently encounters the skeletons of degenerated organisms, which were no doubt introduced into the culture with the inoculation material and died afterwards. In regard to their general appearance there is a striking contrast between the spirochætæ found in the blood of an infected mouse or rat and those seen in young cultures. The former are quite regular in their curves and length. The cultivated organisms are usually irregularly wavy and shorter. The movements are similar in both; at one time there is a serpentine forward movement and at other times there are violent lateral vibrations which involve the middle portion of the body and both ends. In older cultures (six or seven days) the lengths of the organisms are more uniform and approach the standard observed in specimens from the blood, but the curves are still shallower and more irregular. The organisms may be entangled in large masses or may be found in pairs or in chains (figure 14). Some specimens have blunt ends to which a mass of minute refractive granules are attached (figure 16). In this stage every organism is actively motile.

The cultures which have just passed their maximum growth enter a period of decline or degeneration. These cultures contain num-

[17] The addition of several drops of fresh, undefibrinated, normal rat blood favors the growth decidedly.

erous motionless forms which have irregular concentrations of protoplasm at various portions of the spiral skeleton. To many organisms are now attached one or more spherical bodies measuring about 0.7 of a micron in diameter and resembling spores. These bodies are, however, not within the protoplasm, but are attached to the sides of the spirochætæ. Free spherical bodies may also be found. There are many skeletons of spirals which show no longer any masses of protoplasm. Sometimes these degenerated forms are closely bound together by a mass of granular protoplasm that varies in size and form. Occasionally quite normal active spirochætæ are seen.

If left alone the degenerative changes just described proceed rapidly and within three or four days almost no spirochætæ with protoplasm can be found. On the other hand, if fresh tissue and ascitic fluid are added, one can rejuvenate these cultures for two to three days, but under these conditions the vigor of the multiplication is limited and feeble.

Mode of Division.—(Figure 15.) In pure cultures *Spirochæta obermeieri* multiplies by longitudinal division, and possibly also by transverse fission. The longitudinal division occurs in almost every organism whether it be single, paired, or in a chain, and irrespective of the length of the spirochæta. As stated already, the spirochætæ cultivated *in vitro* are less curved than those found in the blood. They are usually somewhat broader, especially when showing longitudinal division. The organisms which are about to divide are somewhat less vigorously motile. The first sign of division is the appearance at any part of the organism of a short narrow cleft parallel to the axis of the spirochæta. Within a few seconds another short cleft comes into view at some distance from the first one; then, after short intervals, a third and fourth cleft are seen. The organism now appears as if its two halves were connected with each other by several protoplasmic bridges of varying width. At the same time each of the halves begins to jerk in a peculiar way, the movements of the halves being alternately in opposite directions. The positions of the several clefts change, one taking the place of another. The fluid character of the protoplasmic bridges which connect the two halves is thus suggested. Finally two clefts fuse

together into a longer cleft, and into this a third and fourth cleft are gradually merged. The organism is now split into two parts, except for the extremities, which in time divide completely. Thus two spirochætæ are formed from one, and, as a rule, one of them dashes away immediately. In some instances the newly formed daughter cells are held together by a thin filament. On the other hand, a transverse division seems also to occur. This mode of division may be indicated by the presence of two or more organisms joined together by a thin filament. It frequently happens that the length of one member of the pair of spirochætæ thus connected is much greater than the other. This appearance suggests that the pair was formed by the transverse division of a longer individual. Nevertheless, the frequency of the occurrence of the transverse fission in culture must be less than that of longitudinal division, because the latter can be seen in any multiplying culture, while an absolutely convincing case of the former has not been observed by me.

Pathogenicity.—The culture of *Spirochæta obermeieri* retains its virulence for rats and mice as was proved by the results of inoculating these animals with the seventh passage *in vitro*. Whether or not the virulence may gradually become attenuated after the cultivation has been continued for a long time will have to be determined later.

PURE CULTIVATION OF SPIROCHÆTA NOVYI.

This variety is now recognized as a distinct species, chiefly through the studies of Fraenkel, Schellack, Uhlenhuth and Haendel, and others. Their conclusions are based upon immunity phenomena, although certain morphological peculiarities are also pointed out. It was first noticed by Schellack that *Spirochæta novyi* resembles *Spirochæta obermeieri* more closely than it does the African varieties, but it is somewhat thinner than the *Spirochæta obermeieri*.

Norris, Pappenheimer, and Flournoy, as well as Novy and Knapp,[18] observed a certain multiplication of this organism in citrated blood kept at body temperature, but a second transfer

[18] In their early articles this variety was dealt with as *Spirochæta obermeieri*, as there was still no differentiation established between these two species. Schellack in 1908 was the first to give this organism the name of *Spirochæta novyi* and separate it from the obermeieri.

showed no further growth. Briefly stated, no culture in the strict sense of the term was obtained.

In my experiment I obtained a growth of this organism in the same kind of medium as that in which the other three strains were cultivated. I have, however, experienced more difficulties with this variety than with the rest, but have at last succeeded in getting it to grow *in vitro*. It is now in its fourth passage.[19]

The general characteristics (figures 18 to 21) of the culture are almost identical with those of *Spirochæta obermeieri* except that the novyi is somewhat thinner than the latter. It shows longitudinal division as well as forms suggestive of transverse division. Up to the present the virulence of the organism growing *in vitro* is comparable to that of the spirochætæ contained in the blood of infected rats and mice.

SUMMARY.

1. A method for the pure cultivation of *Spirochæta duttoni, Spirochæta kochi, Spirochæta obermeieri,* and *Spirochæta novyi* is described in this paper. *In vitro* these strains reach their maximum growth after seven, eight, or nine days at 37° C. For their multiplication they require the presence of a piece of fresh sterile tissue and a body fluid capable of forming a loose fibrin with the tissue. The presence of some oxygen seems indispensable for their growth, since they fail to grow in an atmosphere of hydrogen or *in vacuo*. No growth was obtained at room temperature.

2. From cultures that show a good growth subcultures can be made and the growth can be kept up in this way for many passages.

3. The pathogenicity of these organisms is not lost by cultivation, although there is a tendency for the virulence to become attenuated after growth *in vitro* has continued for a long time.

4. Longitudinal division has been observed and was followed under the dark-field microscope in fresh preparations from cultures. It occurs in all the species irrespective of the length of the organism. Transverse division seems also to occur, but I have not yet observed the entire process.

[19] A more vigorous growth in subcultures can be obtained by adding several drops of fresh, undefibrinated, normal rat blood to the media.

EXPLANATION OF PLATES.

PLATE 19.

Series A.—Schematic reproductions of the spirochætæ under the dark-field microscope.

FIGS. 1 to 5. Different stages of growth of *Spirochæta duttoni* in pure culture. Fig. 1, young forms; Fig. 2, average forms with spore-like bodies in one of the spirochætæ; Fig. 3, a mass of spirochætæ; Fig. 4, longitudinal division; Fig. 5, degeneration forms.

FIGS. 6 to 11. Different stages of growth of *Spirochæta kochi* in pure culture. Fig. 6, young forms; Fig. 7, average forms; Fig. 8, spirochætæ with spore-like bodies; Fig. 9, an entangled mass of the spirochætæ; Fig. 10, longitudinal and transverse division; Fig. 11, degeneration phase.

FIGS. 12 to 17. *Spirochæta obermeieri* in pure culture. Fig. 12, young forms; Fig. 13, usual forms; Fig. 14, a mass of growing spirochætæ; Fig. 15, longitudinal and transverse division; Fig. 16, blunt forms with granulation; Fig. 17, degeneration phase.

FIGS. 18 to 21. *Spirochæta novyi* in pure culture. Fig. 18, young forms; Fig. 19, average forms; Fig. 20, longitudinal and transverse division; also a few spore-like bodies; Fig. 21, degeneration stage.

PLATE 20.

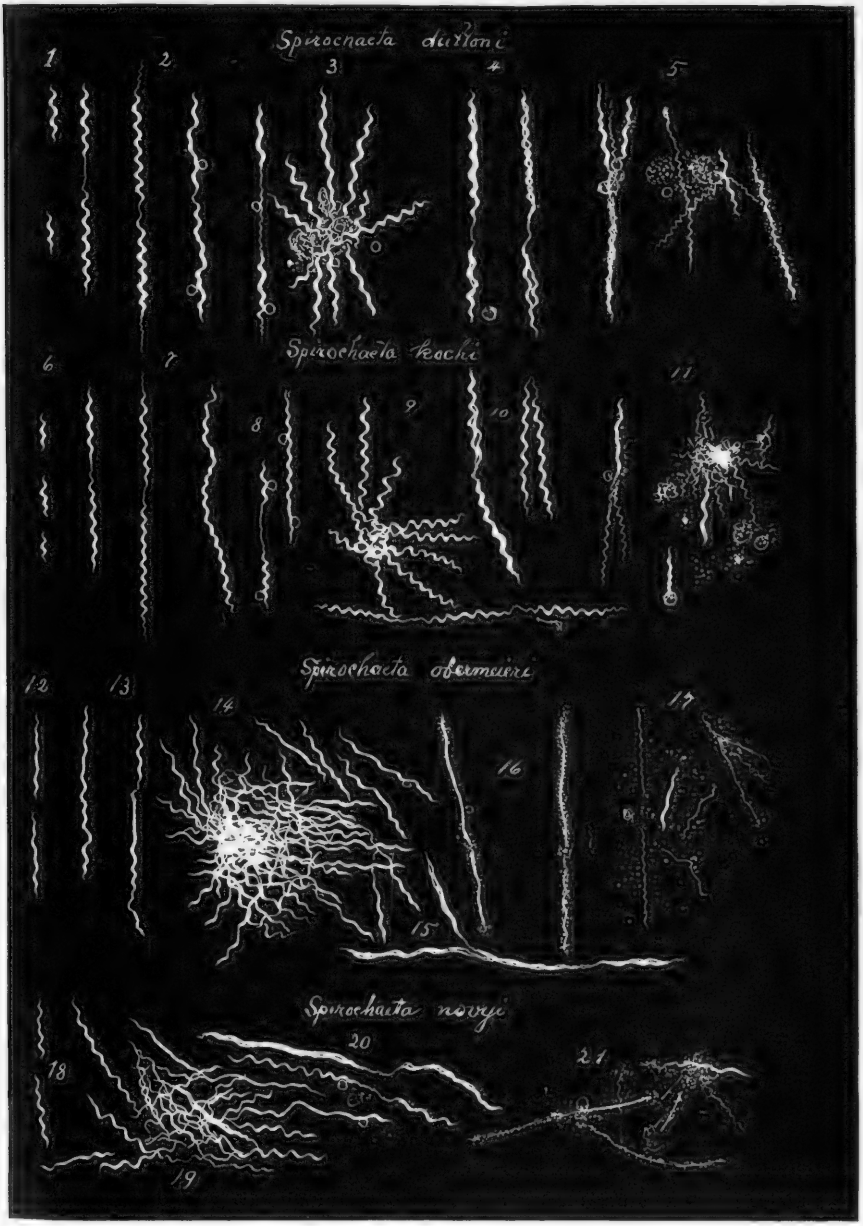
Series B.—Microphotographs of the spirochætæ from the preparations fixed in sublimate alcohol and stained for twelve hours with the Giemsa solution. × 1,100.

FIGS. 22, 23, and 24. *Spirochæta duttoni* from pure cultures (fourth generation). Fig. 22, average forms; Fig. 23, one long and two short forms; Fig. 24, an entangled mass.

FIGS. 25 and 26. *Spirochæta kochi* from pure cultures (twenty-fourth generation). Fig. 25, young forms; Fig. 26, near the time of decline (tenth day).

FIGS. 27, 28, 29, and 30. *Spirochæta obermeieri* from pure cultures (fourth generation). Fig. 27, an average and a small form; Fig. 28, a doubled-up form; Fig. 29, entangled spirochætæ near the time of decline (eighth day); Fig. 30, a peculiar form consisting of partially fused spirochætæ, probably a result of imperfect longitudinal division in succession. This often occurs in a less favorable culture medium. It is not an artifact produced during the fixation, because it can be seen in a fresh preparation under the dark-field microscope; each individual of the mass is actively motile.

FIGS. 31 and 32. *Spirochæta novyi* from pure cultures (fourth generation). Fig. 31 shows the average forms, and Fig. 32 shows a doubled-up specimen.


Spirochaeta duttoni
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Spirochaeta kochi
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Spirochaeta obermeieri
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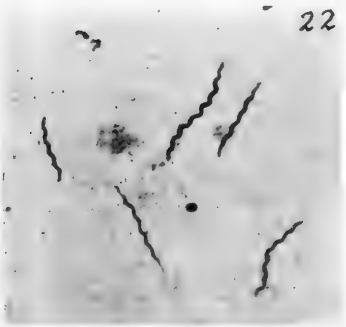
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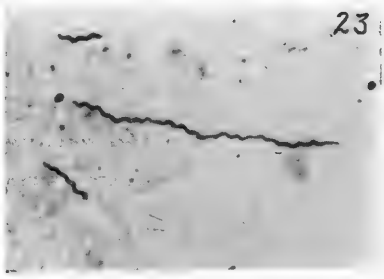
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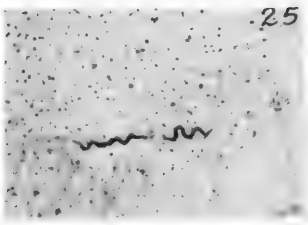
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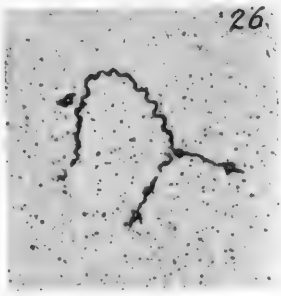
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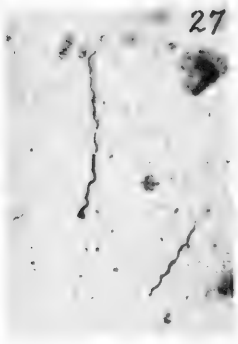
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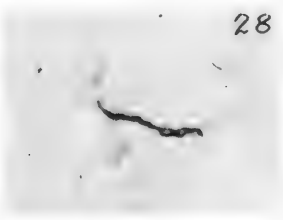
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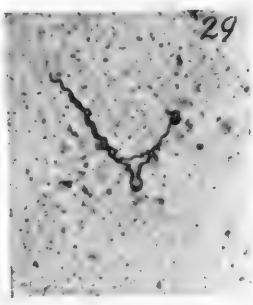
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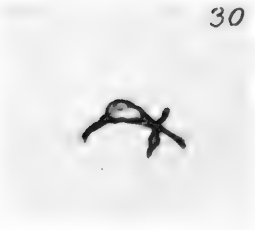
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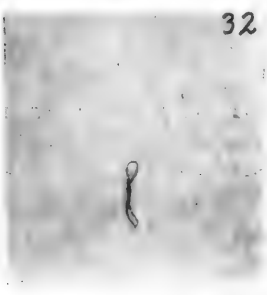

32

A METHOD FOR CULTIVATING TREPONEMA PALLIDUM IN FLUID MEDIA.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

Treponema pallidum is an obligatory anaerobe which together with certain other spirochætæ forms a special class of organisms whose growth requires the presence of a fresh, sterile tissue in culture media.[1] The growth of *Treponema pallidum* in any suitable medium is very slow, and continues for a considerable length of time. In a solid medium, for which the pallidum shows a particular preference, the growth continues for about two months after transplantation. On the other hand, the cultivation of the pallidum in a fluid medium, with the addition of fresh tissue, by means of an anaerobic jar in which oxygen is removed through the combination of vacuum, displacement with hydrogen and absorption by pyrogallol, has given rather inconstant results. In one series of cultivations the growth may be quite favorable, while in others it may fail altogether. The loss of time and material from unsuccessful fluid cultivations is very great and the entire procedure is therefore in need of improvement.

The method which I am about to describe in detail has already proved to be entirely satisfactory and possesses certain special features that are lacking in the usual anaerobic cultural methods now in general use. In the case of the usual method, an ordinary test-tube is filled with the fluid in which the cultivation of an organism is intended, the fluid is then inoculated, and cultivated in a sealed jar under anaerobic conditions. The process is quite satisfactory for the cultivation of most anaerobic bacteria, but, as has already been mentioned, it is unreliable for obtaining growth of the

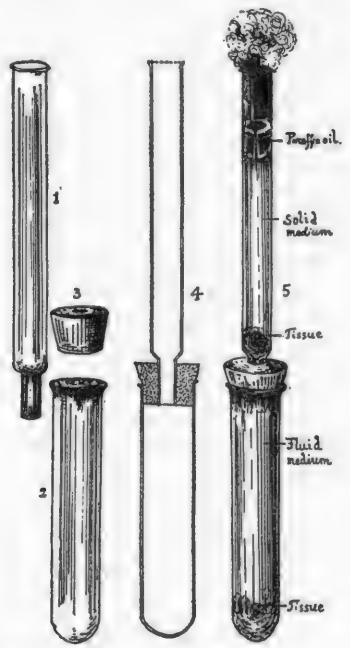
[1] It may be stated that the addition of sterile fresh tissue to a fluid medium alone is not only inadequate to make the pallidum grow, but is unable to keep it alive for any length of time. In this respect the pallidum is very different from the obligatory anaerobic bacteria, as the latter grow abundantly in such a medium.

pallidum in a fluid medium even with the addition of fresh tissue. The failure to effect cultivation of the pallidum by this method is doubtless due to the fact that with this process the removal of oxygen is often too incomplete to enable the inoculated pallidum to grow or even to survive the time required by the fresh tissue to absorb the remaining oxygen and render the fluid perfectly anaerobic. In other words, *Treponema pallidum* is too delicate an organism to survive the unfavorable conditions which last for some time within the fluid in which it is freshly transplanted. The source of this difficulty can, however, be removed by providing the organism with a more suitable medium in which it can at once begin to adjust itself. A solid medium consisting of a mixture of ascitic fluid and agar with the addition of a fresh, sterile tissue fulfills this requirement. Thus the new method which is designed to exclude this difficulty is composed of two distinct parts; one for the solid and the other for the fluid cultivation. By combining both the solid and the fluid culture media for simultaneous cultivation, the shortcoming of the latter is easily supplemented by the former which is highly suitable for the growth of the pallidum. By this method the pallidum grows at first in the solid medium as usual, and then, when the cultural conditions in the fluid portion become favorable, it migrates from the solid portion into the fluid and multiplies there abundantly.

A solid medium in this method not only serves as a reservoir of the growing spirochætæ for the fluid culture, but it is also a good indicator for determining the purity of the culture; for should any contamination occur, it is infallibly indicated by the development of the colonies of bacteria in the agar column, while it is quite difficult to detect a contamination with certain bacteria in a fluid medium alone. As will be seen later, the entire process of cultivation is carried on without the aid of a sealed jar, vacuum, or pyrogallol. The state of growth, or any changes that may take place in the medium, can easily be observed at any time without disturbing the cultivation, and, if desired, the content of the culture may, under aseptic precautions, be drawn out and examined. These are the features which may be regarded as special advantages of the new process in comparison with the older procedures.

METHOD.

Construction of the Culture Tube.—This consists of a test-tube, 1.7 centimeters wide and 20 centimeters long, whose bottom ends in a hollow projection made by fusing a short piece of a strong glass tubing, 0.7 of a centimeter bore, to the perforated bottom (text-figure, 1); a large test tube, 2.5 centimeters wide and 15 centimeters long (text-figure, 2); and a perforated rubber cork, size 5 (text-figure, 3). These parts are combined by connecting the two tubes by means of the rubber cork (text-figure, 4). The



TEXT-FIG. For description see text, p. 213.

upper tube is intended for the solid, and the lower for the fluid culture medium.

Preparations for Cultivation.—The double tube just described is thoroughly cleaned and dried; the upper tube is then plugged with non-absorbent cotton. Just before use the whole is sterilized in an

autoclave. In the meantime pieces of sterile, fresh rabbit kidney are prepared. A slightly alkaline agar (2 per cent.) is also freshly prepared, autoclaved, and kept in a fluid state. A sufficient quantity of sterile paraffin oil should be kept on hand.

Process of Cultivation.—1. After the double tube cools, one or two pieces of the sterile, fresh tissue are put into the lower tube. This is done by removing the rubber cork and the upper tube together, which are quickly replaced and fitted as tightly as possible. These parts must not be loosened again. Another piece of tissue is now put into the upper tube. The tissue goes down to the perforated bottom of the tube, but it should be large enough not to pass through the connecting hole into the lower tube.

2. After tissues have been placed in both tubes, the lower tube is filled with ascitic fluid or a mixture of ascitic fluid and bouillon. This is done by means of a large, sterile bulb pipette whose tubular portion is so drawn out as to pass through the connecting tube with a margin of space that permits the air that is displaced by the fluid to escape. The lower tube must be filled up until there is no air bubble inside the tube.

3. The inoculation of the pallidum is next made. By means of a long capillary pipette, made by drawing a sterile glass tubing, a sufficient quantity of a well growing culture is aspirated and used for inoculation of the double tube. For this purpose a syringe with a connecting pressure rubber tubing is recommended. First the capillary pipette is inserted into the fluid of the lower tube into which some of the contents are forced; then the remaining portion of the culture in the pipette is emptied into the upper tube just about the tissue.

4. When the inoculation has been performed, the upper tube is filled with a solid medium. The medium is prepared by mixing one part of ascitic fluid with two parts of slightly alkaline agar in a sterile flask. The mixing is done when the temperature of the melted agar is about 42° C. Before solidification of the ascitic agar sets in, the medium is quickly distributed into the upper tube. The quantity of the medium is about fifteen cubic centimeters.

5. The last step is to add an arbitrary amount of sterile paraffin

oil in order to cover the surface of the solid medium. About three cubic centimeters suffice (text-figure, 5).

6. The culture is then incubated at 37° C.

For the sake of clearness I have described the procedure as if I were dealing with a single set of double tubes, but in practice it is my custom to employ twelve sets at a time. The advantages of using several sets at a time are twofold. The first is an economic advantage: when one kidney is removed from a normal rabbit it usually furnishes enough tissue to fill at least six sets. As I usually sacrifice one rabbit, I have enough tissue for a dozen sets. The second advantage in making several sets is that, owing to the numerous steps that must be taken in making one series of cultivation, the possibilities of contamination are very great and it is best to provide against this accident so as to insure success.

As regards the capacity of the culture tubes, it is understood that it depends altogether upon the purpose of the work and whether or not a large quantity of the culture is desired. As the nature of my work requires as large a quantity of the material as I can obtain, I use comparatively large tubes.[2] But, according to my experience, it is not advisable to employ still larger tubes, as the result may be an enormous waste of material when accidental contamination takes place.

The method described in this article is especially adapted for the pure cultivation of *Treponema pallidum, Treponema microdentium, Treponema macrodentium, Treponema refringens, Treponema mucosum,* and *Treponema pertenue* in fluid media, when these organisms have been growing already in solid media. It is unsuitable for obtaining the growth of these spirochætæ when they are associated with other bacteria.

SUMMARY.

1. A method is described for obtaining pure cultures of *Treponema pallidum* and allied species of spirochætæ in fluid media.

2. The principle of the method is based upon the superposition of a favorable culture medium upon a less favorable one.

3. The method here described is not suited to the growth of spirochætæ when they are admixed with contaminating bacteria.

[2] The fluid culture of *Treponema pallidum* is used for the preparation of luetin as well as for immunity studies.

THE PREVENTIVE ACTION OF DIPHTHERIA ANTITOXIN IN SERUM-SENSITIZED RABBITS.*

By PAUL A. LEWIS, M.D.

(*From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.*)

A number of years ago it became apparent that antibodies were produced by the injection into mammals of most foreign proteids. It was soon learned that these antibodies were either themselves proteids or were very closely attached to certain of the serum constituents of proteid nature, but efforts to separate the antibodies from the proteid constituents of the serum have not yet succeeded.

The probability that the antibodies are proteid in nature has given rise to many attempts to produce, in turn, specific reaction bodies against the antibodies. As a result it has been concluded that in the case of the simpler forms, those belonging to the first group of Ehrlich,—the agglutinins, the precipitins, and the true antitoxins,—no such antibodies can be formed.

It would be in accord with this conclusion to suppose, for example, that if diphtheria antitoxin were repeatedly administered to an individual over considerable periods of time, the antitoxin of each fresh injection would be absorbed and eliminated exactly as in the case of the first injection. Studies in anaphylaxis have, however, so broadened our point of view that we may recognize it as a law that an animal once sensitized with a foreign proteid of a certain high grade of complexity will never react normally to the same proteid again. Furthermore our conception of the relationships existing in complex mixtures of proteids has become somewhat better defined and we no longer look for perfectly clean cut reactions confined to a single factor in such a mixture, but expect that related factors will be more or less involved.

* Read before the American Association of Pathologists and Bacteriologists, April 6, 1912. Received for publication, April 17, 1912.

From a theoretical point of view, therefore, it has again become a legitimate question whether diphtheria antitoxin, and in fact antitoxins in general, are proteids coming within the range of these rules. The extended use of antitoxin in practice makes it doubly important to have precise knowledge of any condition which may affect its activity.

As the result of experiments which began with those of von Dungern,[1] we know that serum proteids (at least those portions having antigenic properties) when injected intravenously into rabbits can be recognized in the blood for a number of days. As successive injections of serum are given, the time during which the proteid can be recognized becomes considerably shortened, presumably because of an increase in the rate of destruction or binding of the proteids.

The experiments I wish to report were based entirely on this observation. Rabbits were given repeatedly subcutaneous injections either of diphtheria antitoxin or of normal horse serum, until they became distinctly hypersensitive. The Arthus phenomenon was the criterion of the hypersensitive state. When definitely sensitized, the animals were given intravenously a determined dose of diphtheria antitoxin and this was followed after an interval by the injection intravenously of a multiple of the lethal dose of diphtheria toxin. The experiments are of course entirely quantitative, and the relationships are best expressed in the tables that follow.

The diphtheria toxin used was a very old test toxin kindly given me several years ago from the Massachusetts State Antitoxin Laboratory by Dr. Theobald Smith. The antitoxin, also old, was from the same source. It is a native low grade serum (approximately 100 units per cubic centimeter, not concentrated). A considerable number of animals was required to make the preliminary determinations of the strength of the toxin and antitoxin. For this portion of the work, animals which had been inoculated on the cornea with tubercle bacilli were used. In most cases the disease had not generalized. The results thus obtained were in all instances controlled by experiments on normal animals and were found to be accurate. Normal animals were, of course, used for the sensitization experiments.

[1] von Dungern, E., Die Antikörper, Jena, 1903.

RESULTS.

The experiments presented in tables I and II show that the certainly fatal dose of toxin 15 C for rabbits, when given intravenously, is 0.03 of a cubic centimeter, or perhaps somewhat less. Against 0.2 of a cubic centimeter of this toxin, or more than six minimal fatal doses, 0.03 of a cubic centimeter of the antitoxin given one hour before the toxin protects perfectly. Protection is also complete with 0.04 of a cubic centimeter of serum given twenty-four hours before the toxin.

TABLE I.

Toxicity of Diphtheria Toxin 15 C for Rabbits.

No. of rabbit.	Date of inoculation.	Dose in c.c.	Result.
161	Nov. 4, 1911	0.01	Sick, recovered.
173	Nov. 4, 1911	0.01	Sick, recovered.
168	Nov. 4, 1911	0.02	Sick, recovered.
169	Nov. 4, 1911	0.02	Sick, recovered.
176	Nov. 4, 1911	0.03	Dead in 48 hrs. ±
171	Nov. 4, 1911	0.03	Dead in 48 hrs. ±
175	Nov. 4, 1911	0.04	Dead in 48 hrs. ±
174	Nov. 4, 1911	0.04	Dead in 48 hrs. ±
186	Nov. 4, 1911	0.05	Dead in 48 hrs.
179	Nov. 4, 1911	0.05	Dead in 36 hrs. ±
183	Nov. 4, 1911	0.10	Dead in 36 hrs. ±
190	Nov. 4, 1911	0.10	Dead in 36 hrs. ±
196	Nov. 7, 1911	0.20	Dead in 36 hrs. ±
200	Nov. 7, 1911	0.20	Dead in 36 hrs. ±
226	Dec. 12, 1911	0.20	Dead in 36 hrs. ±
231	Dec. 12, 1911	0.20	Dead in 36 hrs. ±
295	Dec. 30, 1911	0.20	Dead in 18 hrs.
273	Dec. 22, 1911	0.20	Dead in 19 hrs.
254	Dec. 22, 1911	0.20	Dead in 19 hrs.
276	Mar. 23, 1911	0.20	Dead in 36 hrs. ±

The experiments presented in table III show that in rabbits previously treated with normal horse serum or with diphtheria antitoxin, neither the dose of 0.03 of a cubic centimeter of antitoxin one hour before nor of 0.05 of a cubic centimeter of antitoxin twenty-four hours before the toxin dose, show any protective influence whatever. By referring to table II, it will be seen that with an interval of one hour 0.03 of a cubic centimeter of serum protected perfectly, that 0.02 of a cubic centimeter protected partially, and that 0.01 of a cubic centimeter of serum gave no definite protective influence. In the sensitized rabbit the protective influence of 0.03 of a cubic

TABLE II.

Protective Action of Diphtheria Antitoxin in Normal Rabbits.

No. of rabbit.	Date of inoculation.	Antitoxin dose in c.c.	Interval.	Toxin dose in c.c.	Result.
227	Dec. 12, 1911	0.01	1 hr.	0.20	Dead in 36 hrs. ±
230	Dec. 12, 1911	0.01	1 hr.	0.20	Dead in 36 hrs. ±
201	Nov. 7, 1911	0.01	1 hr.	0.20	Dead in 36 hrs. ±
202	Nov. 7, 1911	0.01	1 hr.	0.20	Dead in 36 hrs. ±
232	Dec. 12, 1911	0.02	1 hr.	0.20	Well.
228	Dec. 12, 1911	0.02	1 hr.	0.20	Dead in 4 days.
229	Dec. 12, 1911	0.03	1 hr.	0.20	Well.
233	Dec. 12, 1911	0.03	1 hr.	0.20	Well.
293	Dec. 30, 1911	0.03	1 hr.	0.20	Well.
212	Dec. 12, 1911	0.04	1 hr.	0.20	Well.
211	Dec. 12, 1911	0.04	1 hr.	0.20	Well.
203	Dec. 7, 1911	0.05	1 hr.	0.20	Well.
205	Dec. 7, 1911	0.05	1 hr.	0.20	Well.
328	Mar. 22, 1912	0.02	24 hrs.	0.20	Dead in 36 hrs. ±
350	Mar. 22, 1912	0.02	24 hrs.	0.20	Dead in 48 hrs. +
278	Mar. 22, 1912	0.03	24 hrs.	0.20	Dead in 36 hrs. ±
353	Mar. 22, 1912	0.03	24 hrs.	0.20	Sick, recovered.
338	Mar. 22, 1912	0.04	24 hrs.	0.20	Well.
357	Mar. 22, 1912	0.04	24 hrs.	0.20	Well.
214	Dec. 12, 1911	0.05	24 hrs.	0.20	Well.
213	Dec. 12, 1911	0.05	24 hrs.	0.20	Well.
258	Dec. 22, 1911	0.05	24 hrs.	0.20	Well.
280	Dec. 22, 1911	0.05	24 hrs.	0.20	Well.

TABLE III.

Protective Action of Diphtheria Antitoxin in Sensitized Rabbits.

No. of rabbit.	Sensitized with	Antitoxin dose in c.c.	Interval.	Toxin dose in c.c.	Result.
251	N. H. S.[2]	0.03	1 hr.	0.20	Dead in 18 hrs.
248	N. H. S.	0.03	1 hr.	0.20	Dead in 20 hrs.
240	Diph. A. T.[2]	0.03	1 hr.	0.20	Dead in 21 hrs.
238	Diph. A. T.	0.03	1 hr.	0.20	Dead in 20 hrs.
244	Diph. A. T.	0.10	1 hr.	0.20	Well.
247	N. H. S.	0.10	1 hr.	0.20	Well.
252	N. H. S.	0.10	1 hr.	0.20	Well.
245	Diph. A. T.	0.20	1 hr.	0.20	Well.
249	N. H. S.	0.20	1 hr.	0.20	Well.
256	N. H. S.	0.20	1 hr.	0.20	Well.
250	N. H. S.	0.05	24 hrs.	0.20	Dead in 24 hrs.
253	N. H. S.	0.05	24 hrs.	0.20	Dead in 23 hrs.
239	Diph. A. T.	0.05	24 hrs.	0.20	Dead in 25 hrs.
243	Diph. A. T.	0.05	24 hrs.	0.20	Dead in 26 hrs.

centimeter of serum is, therefore, about equal to that of 0.01 of a cubic centimeter to 0.02 of a cubic centimeter of serum injected into a normal rabbit. In other words, in rabbits sensitized to serum,

[2] N. H. S. = normal horse serum; Diph. A. T. = diphtheria antitoxin.

diphtheria antitoxin is but one third to two thirds as efficient as in normal rabbits. These figures are further substantiated by the experiments in which the interval was twenty-four hours. Here the efficiency of 0.05 of a cubic centimeter in the sensitized rabbit was about equal to that of 0.02 of a cubic centimeter to 0.03 of a cubic centimeter of serum in the normal rabbit.

DISCUSSION.

From experiments that are so limited we do not wish to draw conclusions which should affect practice. These results, however, lead naturally to the suggestion that the curve for the absorption and elimination of antitoxin as developed by Henderson Smith[3] should be elaborated to cover the case of the human being who has previously had injections either of diphtheria antitoxin or of normal horse serum. It should be recalled that the marketed antitoxin is now chiefly concentrated globulins, according to Gibson. But the curves of Henderson Smith were prepared before the present method of concentration was generally adopted. The curve for globulins may well differ from that for the native serum.

The evidence obtained points clearly to the conclusion that in a native low grade serum the antitoxin is so related to the proteids that it is involved in the reaction of allergy. As far as the experiments go, the antitoxin does not seem to sensitize against itself, and only a part of it is affected by the second reaction. Antitoxin itself, therefore, may not be a proteid coming within the group which gives rise to an immunity reaction.

It may be added that these results are in general accord with the recently reported experiments of Doerr and Pick[4] on the rate at which the precipitable proteids of horse serum and cholera agglutinin disappear from the circulating blood of sensitized as compared with normal rabbits. The stated conclusions of these authors, however, are not in complete acccord with the experimental evidence presented by them.

[3] Smith, J. Henderson, *Jour. Hyg.*, 1907, vii, 205.
[4] Doerr, R., and Pick, R., *Centralbl. f. Bakteriol., Orig.*, 1912, lxii, 146.

THE SELECTIVE BACTERICIDAL ACTION OF GENTIAN VIOLET.*

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Plates 21–31.

During the course of an experimental study of bacteriuria, specimens of urine thought to contain *Bacillus prodigiosus* which had been injected into the circulation became contaminated with *Bacillus subtilis*. In an effort to rid them of this contamination, the effect on the two organisms of a number of common dyes was studied and a striking selective power possessed by gentian violet was brought to light. It is the purpose of this paper to record the original observations and to report an extension of the study to other bacteria.

In my first experiments the dye was applied directly to the organisms, and its effect was noted after transplanting the stained bacteria to agar. Five drops of a saturated aqueous solution of gentian violet[1] were added to a forty-eight hour broth culture of the organism to be tested. The mixture was shaken well and allowed to stand for one hour. Transplants were then made to agar, a control inoculation of unstained organisms being made at the same time upon agar in a second tube. After twenty-four hours' incubation at 37° C. a striking selective property of the dye had asserted itself. When *Bacillus prodigiosus* was the organism used, no difference was observed between the tube inoculated with the stained, and that inoculated with the unstained organisms; growth was equally luxuriant in both (figure 1). In the case of *Bacillus subtilis*, however, the results were quite different, for the stained *subtilis* failed to grow (figure 2).

* This work was made possible by the financial assistance of Dr. Hugh H. Young. Received for publication, May 6, 1912.

[1] Grübler's gentian violet was used in all the experiments described in this paper.

The *Bacillus prodigiosus* used in the early experiments was a prolific chromogen and was typical in all respects. The *Bacillus subtilis* was isolated from the air and possessed all the characteristics described by Matzuschita for *Bacillus subtilis* (Ehrenberg). In subsequent studies, several strains of each of a number of organisms derived from different sources were employed.

This selective action of gentian violet for *Bacillus subtilis* and *Bacillus prodigiosus* was so specific and constant that it led to a study of the effect of the dye on a number of the more common organisms, and it soon became apparent that bacteria could be divided into two groups by their behavior toward gentian violet. In the group with *Bacillus subtilis* fell *Micrococcus aureus, Micrococcus albus, Bacillus diphtheriæ, Bacillus anthracis, Sarcina rosea, Streptothrix actinomyces,* blastomyces, and certain yeasts. These organisms refused to grow when stained with gentian violet and transplanted to agar. The result, for instance, in the case of *Bacillus subtilis* is shown in figure 2, and in the case of blastomyces in figure 3.

In the group with *Bacillus prodigiosus* fell *Bacillus pyocyaneus, Bacillus typhosus, Bacillus paratyphosus, Bacillus lactis aerogenes,* and *Bacillus suipestifer.* These organisms, after an hour's contact with gentian violet, grew as promptly and as luxuriantly as organisms which had not been exposed to the dye. Figure 1, which shows the result in the case of *Bacillus prodigiosus,* illustrates the behavior of the whole group.

In order to determine whether the stained organisms which failed to grow on slant agar had simply been restrained by the dye, and if so to give them an opportunity to recover, inoculations of stained organisms were made into broth. After incubation for twenty-four hours, agar plates were made from these tubes. The result of such an experiment is shown in figure 4, where it is evident that the plate made from the tube inoculated with *Bacillus subtilis* remained sterile, whereas the other was crowded with colonies of *Bacillus prodigiosus.*

EFFECT OF THE DYE IN MEDIA.

A study was next made of the effect of the dye when added to the media in which the organisms were grown, instead of being

applied directly to the bacteria themselves. In the first experiments the dye was added to broth or agar tubes in dilutions of about 1 to 100,000; and it was found that these tubes when inoculated with the organisms already studied by the method of vital staining, revealed in a similar way the selective property of gentian violet. The parallelism between the behavior of the organisms when stained with gentian violet, and their behavior when grown in gentian violet media, was complete. This observation, made on about thirty of the commoner organisms, makes it probable that any organism which fails to grow when stained, will also fail to grow in media containing the dye; while any organism, the growth of which is unimpeded by the staining, will grow readily in gentian violet media. To this rule there may be exceptions not revealed in the later studies, in which the method of vital staining was abandoned and only the behavior of the organisms in gentian violet media was investigated. But there can be no doubt that the inhibiting action of the gentian violet is more constant when the dye is incorporated with the media, than when it is applied directly to the bacterial bodies.

TECHNIQUE OF DIVIDED PLATES.

In order to facilitate the study of the effect of the dye on large numbers of organisms, grown under conditions rigidly controlled, the method of divided plates was devised. An ordinary Petri dish was divided into two compartments by the insertion of a metal strip. For this purpose Halsted's aneurysm metal was used. It was cut into strips about 0.5 cm. wide, and a little longer than the diameter of the plate, care being taken to have the edges as straight as possible. These strips were then slightly bent so as to form a curve with its convexity toward the right, and placed in the dish with its convexity in the opposite direction, toward the left. The spring thus given the metal held the strip in the dish with sufficient snugness. After sterilization in the Arnold sterilizer the metal slip was readjusted, if necessary, with sterile forceps so as to fit the dish as accurately as possible. Plain agar was then poured into one compartment, the dish being slightly tilted so as to allow the agar to approach the dividing metal slip slowly. No Petri dish is perfectly flat, and accurate contact between the metal slip and the bottom of the dish is not easy to obtain. The small amount of agar, however, which flowed under the metal sealed, on cooling, the interstice between metal and glass and thus provided a water-tight contact. When the plain agar had solidified, gentian violet agar was poured into the opposite compartment. This was allowed to cool and the metal slip was lifted out with sterile forceps. The result was an agar plate sharply divided into two compartments, one containing pure agar and one containing gentian violet agar.

On such plates inoculations of the organisms to be studied were always made

from broth cultures or from salt solution emulsions, and with a loop made of fine platinum wire. These precautions were found essential, for a good result could not be obtained if the agar surface was scratched with a heavy wire, or if the distribution of the organisms was uneven. Moreover, it is essential that the bacteria be in intimate contact with the surface of the agar; for even *B. subtilis,* which never grows when evenly applied to the surface of gentian violet agar from a broth culture, will sometimes develop slightly when the organisms are thickly piled up on the surface, as must happen when the stroke is made from an agar slant.

In making the stroke inoculations, a loop of broth culture is gently rubbed back and forth a number of times over the plain agar side of the plate, the loop being carefully held parallel to the agar surface so as to avoid scratching it. The platinum needle is then sterilized and a second loop is similarly applied to the gentian violet side. In this way an approximately equal number of organisms is placed on the two halves of the plate, an even distribution of the organisms is obtained, and intimate contact between the bacteria and the agar is secured. The smooth track made by the needle, in applying the organisms to the agar surface, is shown on the sterile gentian violet side of the plate (figure 5).

A study by this method of organisms the reaction of which to vital staining with gentian violet had previously been determined, demonstrated that the behavior of an organism on divided plates might be regarded as a cultural characteristic of the most clear cut kind. The bacteria studied reacted in two distinct ways. One group of organisms, consisting of those the growth of which had been stopped when stained by the dye, refused to grow at all on the gentian violet agar; while the members of the other group, consisting of organisms the growth of which had been uninfluenced by the vital staining, grew equally well on the two sides of the plate. This differential reaction, which was very sharp and very constant, is well illustrated in figures 5, 6, 7, and 8. Compare, for instance, figure 5, where *Sarcina rosea* refuses even to approach the boundary line between plain and gentian violet agar, with figure 6, where *Bacillus prodigiosus* grows equally well on the two sides of the plate. Or notice figure 7, where *Bacillus subtilis* is shown flanked by two stroke inoculations of *Bacillus prodigiosus;* or figure 8, where *Micrococcus aureus* is flanked by two stroke inoculations of *Bacillus typhosus;* both photographs illustrate the precision with which the Gram positive organisms are singled out for destruction by the dye, which leaves the growth of the Gram negative organisms undisturbed.

The astonishing sensitiveness of a group of hardy and resistant spore-bearing Gram positive bacteria to the presence of gentian violet is well illustrated in figures 9, 10, and 11, which show the behavior of *Bacillus subtilis, Bacillus mycoides,* and *Bacillus anthracis* on divided plates. The sharp margin of the growth indicates the place where the luxuriant development on the plain agar side suddenly and completely ceases; and this margin, it will be seen, lies at some distance from the dividing line between the two sides of the plate (the "dead line"). In other words, not only do these bacteria refuse to grow on the gentian violet agar; but they also refuse to grow in its neighborhood. This phenomenon is apparently to be explained by the readiness with which the dye diffuses through agar. As a consequence of this diffusion the plain agar just beyond the dead line soon contains sufficient stain to prevent the development of organisms, although it is difficult or impossible to detect its presence by the eye. This is an indication of the extreme dilution in which the dye may be effective. This point will be referred to later.

On the plain agar in the divided plate the margin of the growth is stained at some distance from the gentian violet agar. The fact is striking enough, whether it is to be explained entirely by the physical laws of diffusion through solid media or whether it indicates a vital, active chemotaxis between the bacteria and the stain. Colonies situated eight to ten millimeters from the gentian violet agar are often deeply stained, although the agar about them shows no trace of the dye to the naked eye.

The organisms which grow on the gentian violet agar also take up the stain readily. This fact is apparent in figure 6. Moreover, the organisms so stained retain their viability. I have transplanted, for instance, *Bacillus typhosus* (which had become deeply stained after growing for three weeks on gentian violet agar), and found that it developed promptly and luxuriantly. How much of the stain is actually within the bacterial bodies, it is difficult to say; but it is certain that smears from such deeply stained growths show the individual bacteria to be rather less intensely colored than would be expected from the macroscopic appearance. This suggests that some of the stain, at least, lay around and not actually within the microörganisms.

By the method of inoculation on divided plates the original study of the commoner organisms was extended to include 334 strains, representing 138 distinct bacterial species.[2] The results showed, as the original observations had suggested, that bacteria could be pretty sharply divided into two groups according as their growth was or was not inhibited by the dye. The findings for each organism are recorded in table I.

It is not expected, of course, that this preliminary survey will need no revision. In the case of the more familiar organisms, authentic strains from various sources have been studied and large numbers of transplants made; and for these the reaction toward gentian violet may be stated with certainty. Where only one strain has been studied, however, and only a small number of transplants have been made, the statement of the violet reaction must be regarded as tentative. Still, in the case of any given species, the results obtained by subsequent study of numerous strains have usually confirmed the original observations for that species; so that the revision necessary in the table ought not to be extensive. It should be stated that it has been impossible to identify all the stock organisms studied, and modifications in the table may be necessary if a few of the stock cultures with which I worked prove not to have been true to name.

PARALLELISM WITH THE GRAM STAIN.

The parallelism of this reaction with the Gram stain, suggested by the earlier observations, was confirmed by more extensive study. If organisms whose growth is inhibited by gentian violet be spoken of briefly as violet positive, and those whose growth is uninfluenced by the dye as violet negative, it is found that the large majority of Gram positive organisms are also violet positive, and the large majority of Gram negative organisms are also violet negative. The actual figures are as follows:

[2] This was made possible through the kindness of Professor C.-E. A. Winslow, who allowed me unrestricted use of the stock cultures at the American Museum of Natural History, and gave me the privileges of his laboratory.

		Violet positive.	Violet negative.
130 species	Gram positive, 77	70 (90+%)	7 (9+%)
	Gram negative, 53	8 (15+%)	45 (84.9+%)

		Violet positive.	Violet negative.
318 strains	Gram positive, 182	165 (90%)	17 (9.3%)
	Gram negative, 136	15 (11%)	121 (88.9%)

The more important Gram positive organisms which do not follow the rule are: *Bacillus welchii* (whose growth may be hindered, but is never stopped, save by extremely strong dilutions); *Bacillus sporogenes;* an occasional strain of streptococcus and of pneumococcus; and most of the acid-fast group, the growth of which is often delayed, but is seldom stopped.

As will be seen in the table, there are also a number of Gram negative organisms which do not follow the rule.[3]

This interesting parallelism between the Gram reaction and the violet reaction should be noticed, but not unduly stressed. The exceptions in each group are too numerous, and the results of the Gram stain itself too notoriously uncertain, to warrant the conclusion from this observed parallelism that the designation "Gram positive" or "Gram negative" really indicates a more fundamental characteristic of bacteria than had hitherto been supposed, and depends on a bond between the organism and the dye of an intimate and significant sort.

It is quite probable that my statistics as regards this parallelism will be modified by further study. In numerous instances I have found great difficulty in deciding, even after repeated stains, whether to classify an organism as Gram positive or negative; and a few discrepancies will be found in the table between the commonly accepted statements on this point and my findings.

CONSTANCY OF BEHAVIOR TOWARD GENTIAN VIOLET.

The behavior of bacteria on divided gentian violet plates is so constant and so clear cut that it must be regarded as a fundamental biological characteristic. A few species have been encountered, like

[3] Observations recently made by Winslow indicate that a fairly large number of Gram negative cocci fail to grow in gentian violet agar.

the streptococcus, an occasional strain of which varies in this respect; but for the vast majority of bacterial species, and for the vast majority of bacterial strains, the behavior toward gentian violet is almost, if not absolutely constant. As regards precision and constancy there can be no comparison whatever between this reaction and the Gram stain; and the violet reaction is further distinguished from the Gram stain by the fact that an organism reacts in the same way toward the dye, no matter what its age. For these reasons it may prove of value in identifying bacteria. Several instances in which the observations have been practically applied in bacteriological studies, will be referred to later.

The effect of the dye is not quite so constant when, instead of being incorporated in the media, it is applied to the bacteria themselves. When thus stained the violet positive organisms almost never grow on agar, but an occasional vegetative cell or spore may escape the dye and develop slowly. I have seen, for instance, an agar slant stroked with stained *Bacillus subtilis* remain sterile for several days, and then one small colony slowly develop.

In broth the results are less constant. *Bacillus subtilis,* stained for one hour and then put into a tube containing six cubic centimeters of bouillon, almost never grows, but if put into a large flask of broth it not infrequently develops. This apparent contradiction is undoubtedly explained in part by the fact that enough of the dye is carried over by the oese in making the transplant to render a relatively small amount of broth sufficiently strong in gentian violet to prevent the growth of bacteria which have previously been injured by staining, for I have found that even in dilutions of 1 to 1,000,000 the dye has a power of this kind.

Whether or not the stained organisms will subsequently develop depends, too, to some extent on the character of the media into which they are transplanted. This fact was demonstrated by the following experiment. Pneumococci which had been trained to grow well on plain agar were stained and planted on this media. They refused to grow. When stained and planted on blood agar, however, they grew in fair luxuriance.

TABLE I.

The Behavior of Organisms Toward Gentian Violet. Organisms Marked + in the Gentian Violet Column are Those the Growth of Which Was Inhibited by the Dye.

Bacterial species.	Bacterial strains.	Gram.	Gentian violet.
1. B. subtilis (8 strains)	1. N. Y..... 106	+	+
	2. N. Y..... 228	+	+
	3. N. Y..... 248	+	+
	4. N. Y. (B. subtilis ruber).. 284	+	+
	5. N. Y..... 357	+	+
	6. J.H.U.	+	+
	7. Isolated from air	+	+
	8. Contamination	+	+
2. B. anthracis (11 strains)	9. N. Y..... 28	+	+
	10. N. Y..... 107	+	+
	11. N. Y..... 108	+	+
	12. N. Y..... 109	+	+
	13. N. Y..... 39	+	+
	14. N. Y..... 43	+	+
	15. N. Y..... 58	+	+
	16. N. Y..... 316	+	+
	17. N. Y..... 317	+	+
	18. N. Y..... 341	+	+
	19. Cornell Med. School	+	+
3. B. pseudoanthracis	20. N. Y..... 342	+	+
4. B. anthracoides, 2 strains	21. N. Y..... 244	+	+
	22. N. Y..... 405	+	+
5. B. megatherium, 2 strains	23. N. Y..... 251	+	+
	24. N. Y..... 270	+	+
6. B. mycoides, 2 strains	25. N. Y..... 115	+	+
	26. N. Y..... 249	+	+
7. B. liodermos	27. N. Y..... 501	+	+
8. B. mesentericus	28. N. Y..... 194	+	+
9. Actinomyces (11 strains)	29. Boston (Wright)	+	+
	30. J.H.U., A. bovis	+	+
	31. J.H.U., A. asteroides	+	+
	32. N. Y..... 148	+	+
	33. N. Y..... 145	+	+
	34. N. Y..... 49	+	+
	35. N. Y..... 147	+	+
	36. N. Y..... 149	+	+
	37. N. Y..... 50	+	+
	38. N. Y..... 146	+	+
	39. N. Y..... 404	+	+
10. Blastomyces (3 strains)	40. Isolated from human case (Welch)	+	+
	41. Isolated from human case (Gilchrist)	+	+

Bacterial species.	Bacterial strains.	Gram.	Gentian violet.
76. B. coli communis, 3 strains	174. N. Y..... 57	0	0
	175. J.H.U.	0	0
	176. J.H.U.	0	0
77. B. capsulatus mucosus, 3 strains	177. N. Y..... 40	0	0
	178. N. Y..... 241	0	0
	179. N. Y..... 468	0	0
78. B. pullorum, 2 strains	180. N. Y..... 277	0	0
	181. N. Y..... 403	0	0
79. B. pneumoniæ, 3 strains	182. N. Y..... 27	0	0
	183. N. Y..... 88	0	0
	184. N. Y..... 343	0	0
80. B. murium, 2 strains	185. N. Y..... 15	0	0
	186. N. Y..... 237	0	0
81. B. alkaligenes, 2 strains	187. N. Y..... 133	0	0
	188. N. Y..... 439	0	0
82. B. fuchsinus, 2 strains	189. N. Y..... 170	0	0
	190. N. Y..... 171	0	0
83. B. icteroides	191. N. Y..... 235	0	0
84. B. bruxellensis	192. N. Y..... 331	0	0
85. B. rosaceus metalloides	193. N. Y..... 318	0	0
86. B. frosti	194. N. Y..... 300	0	0
87. B. coscoroba	195. N. Y..... 436	0	0
88. B. fluorescens	196. N. Y..... 29	0	0
89. B. liquefaciens	197. N. Y..... 31	0	0
90. B. coli communior A_1	198. N. Y..... 139	0	0
91. B. coli communior A_1 A_2 B C (4 strains)	199. N. Y..... 123	0	0
	200. N. Y..... 19	0	0
	201. N. Y..... 144	0	0
	202. N. Y..... 137	0	0
B. coli (rubra) varietas	203. N. Y..... 328	0	0
B. coli (lutea) varietas	204. N. Y..... 329	0	0
92. B. paracoli (5 strains)	205. N. Y..... 455	0	0
	206. N. Y..... 452	0	0
	207. N. Y..... 8	0	0
	208. N. Y..... 17	0	0
	209. N. Y..... 30	0	0
93. B. typhosus	210. J.H.U. Stock	0	0
	211. Isolated from human case (Bischoff)	0	0
	212. Isolated from human case (Goldmann)	0	0
	213. Isolated from human case (Scott)	0	0
	214. Isolated from human		

	case (Welch)	+	+		case (Goldmann)	0	0
3 strains	41. Isolated from human case (Gilchrist)	+	+		213. Isolated from human case (Scott)	0	0
	42. Isolated from guinea pig (Austrian)	+	+		214. Isolated from human case (Rosis)	0	0
11. M. aureus	43. N. Y..... 312	+	+		215. Isolated from human case (Brewer)	0	0
	44. Isolated from rabbit, septicemia	+	+		216. Isolated from human case (Gunther)	0	0
5 strains	45. Isolated from human blood	+	+	14 strains	217. Isolated from human case (Kane)	0	0
	46. J.H.U.	+	+		218. Isolated from human case (Shephard)	0	0
	47. J.H.U.	+	+		219. Isolated from human case (Christensen)	0	0
12. M. albus, 2 strains	48. J.H.U.	+	+		220. Isolated from human case (Buchanan)	0	0
	49. J.H.U.	+	+		221. Isolated from human case (Lolopsky)	0	0
13. Micrococcus (isolated from human sputum; similar to M. catarrhalis, but Gram positive), 3 strains	50. From human sputum (Smith)	+	+		222. Isolated from human case (Barrett)	0	0
	51. From human sputum (Siegelmann)	+	+		223. Isolated from human case (Tuohy)	0	0
	52. From human sputum (Mercudas)	+	+				
14. M. citreus, 2 strains	53. N. Y..... 354	+	+				
	54. N. Y..... 458	+	+				
15. M. citreus agilis	55. N. Y..... 466	+	+				
16. M. agilis	56. N. Y..... 488	+	+	94. B. paratyphosus	224. N. Y..... 323	0	0
17. M. versatilis	57. N. Y..... 430	+	+		225. Schottmüller	0	0
18. M. luteus	58. N. Y..... 272	+	+		226. Cushing	0	0
19. M. flavus	59. N. Y..... 279	+	+		227. J.H.U. (Bodash)	0	0
20. M. aurantiacus	60. N. Y..... 467	+	+		228. J.H.U. (Kurth)	0	0
21. M. tetragenus	61. N. Y..... 209	+	+		229. J.H.U. (Hicks)	0	0
22. M. ascoformans	62. N. Y..... 457	+	+		230. Isolated, patient (Johnson)	0	0
23. M. neoformans	63. N. Y..... 260	+	+		231. Isolated, patient (Elliott)	0	0
24. M. ureæ, 2 strains	64. N. Y..... 130	+	+		232. Isolated, patient (Vernon)	0	0
	65. N. Y..... 464	+	+		233. Isolated, patient (Purnell)	0	0
25. M. rhodochrous	66. N. Y..... 480	+	+		234. Isolated, patient (Snyder)	0	0
26. Rhodococcus	67. N. Y..... 217	+	+		235. Isolated, patient (Cheek)	0	0
27. M. candicans	68. N. Y..... 471	+	+		236. Isolated, patient (Taylor)	0	0
28. M. catarrhalis[4]	69. N. Y..... 437	+	+		237. Isolated, patient (Scott)	0	0
29. Sarcina lutea	70. N. Y..... 208	+	+	18 strains	238. Isolated, patient (Travathin)	0	0
4 strains	71. N. Y..... 216	+	+		239. Isolated, patient (Mannigel)	0	0
	72. N. Y..... 314	+	+		240. Isolated, patient (Westerkamp)	0	0
	73. N. Y..... 345	+	+		241. J.H.U. Gwynn	0	0
30. Sarcina rosea, 3 strains	74. N. Y..... 462	+	+		242. J.H.U.	0	0
	75. Isolated from air	+	+	95. Vibrio choleræ	243. N. Y..... 296	0	0
	76. Isolated from sputum	+	+	5 strains	244. N. Y..... 298	0	Delay 0
31. Sarcina flava	77. N. Y..... 481	+	+		245. N. Y..... 299	0	Delay 0
32. Sarcina aurantiaca, 3 strains	78. N. Y..... 315	+	+				
	79. N. Y..... 474	+	+				
	80. N. Y..... 3	+	+				
33. Sarcina ventriculi	81. N. Y..... 479	+	+				
34. Sarcina mobilis	82. N. Y..... 482	+	+				
35. Sarcina (variety?)	83. Isolated from air, N. Y..... 101	+	+				
36. Mould (reddish), 2 strains	84. Air contamination	+	+				
	85. Air contamination	+	+				
37. B. sorbose	86. N. Y..... 10	+	+				
38. Azoobacter.							

	beijerincksi	87. N. Y.	111	+	+				
39. B. oxalaticus		88. N. Y.	118	+	+	96. B. choleræ suis	247. J.H.U.	o	o
40. B. butyricus		89. N. Y.	56	+	+		248. N. Y.	239	o	o
41. B. arborescens[5]		90. N. Y.	143	+	+		249. N. Y.	258	o	o
42. B. lactis (V Flügge)		91. N. Y.	181	+	+	6 strains	250. N. Y.	332	o	o
43. B. lactis (VI Flügge)		92. N. Y.	182	+	+		251. N. Y.	333	o	+
44. B. kiliensis		93. N. Y.	183	+	+		252. N. Y.	334	o	o
45. B. balticus		94. N. Y.	187	+	+	97. B. dysenteriæ,	253. Shiga		?	o
46. B. sternbergii		95. N. Y.	232	+	+	3 strains	254. His-Russell		?	o
47. B. amylovorus		96. N. Y.	303	+	+		255. N. Y.	121	?	Delay o
48. B. carotarum		97. N. Y.	304	+	+	98. B. enteritidis	256. N. Y.	18	o	o
49. B. plicatus[6]		98. N. Y.	356	+	+		257. N. Y.	25	o	o
50. B. ramosus		99. N. Y.	361	+	+	5 strains	258. N. Y.	26	o	o
51. B. radicicola		100. N. Y.	374	+	+		259. N. Y.	132	o	o
52. Cladothrix dichotoma		101. N. Y.	432	+	+		260. N. Y.	234	o	+
53. B. buccalis		102. N. Y.	443	+	+		261. J.H.U.		o	o
54. Mycobact. rhusiopathæ		103. N. Y.	456	+	+	99. B. lactis aerogenes	262. N. Y.	136	o	o
							263. N. Y.	122	o	Delay o
55. B. javaniensis		104. N. Y.	472	+	+		264. N. Y.	180	o	o
56. B. zopfii		105. N. Y.	475	+	+	7 strains	265. N. Y.	445	o	o
57. B. amylobacter		106. N. Y.	476	+	+		266. N. Y.	448	o	o
58. Streptococcus		107. N. Y.	6	+	+		267. N. Y.	126	o	o
		108. N. Y.	9	+	+		268. N. Y.	273	o	o
		109. N. Y.	12	+	+	100. Microspira protea,	269. N. Y.	336	o	o
		110. N. Y.	32	+	?	3 strains	270. N. Y.	440	o	o
		111. N. Y.	35	+	+		271. N. Y.	223	o	+
		112. N. Y.	222	+	+	101. Microspira metchnikovii, 2 strains	272. N. Y.	224	o	o
		113. N. Y.	265	+	+		273. N. Y.	337	o	+
		114. N. Y.	266	+	+	102. Microspira tyrogena, 2 strains	274. N. Y.	225	o	Delay o
		115. N. Y.	309	+	+		275. N. Y.	338	o	+
		116. N. Y.	310	+	?	103. Microspira schuylkilliensis	276. N. Y.	442	o	+
		117. N. Y.	311	+	+					
		118. N. Y.	346	+	+	104. Microspira berolinensis	277. N. Y.	461	o	+
		119. N. Y.	386	+	+					
		120. N. Y.	387	+	+	105. Microspira danubica	278. N. Y.	463	o	o
		121. N. Y.	388	+	+	106. Microspira phosphorescens	279. N. Y.	465	o	o
		122. N. Y.	390	+	+					
		123. N. Y.	412	+	+	107. B. astheniæ[7]	280. N. Y.	103	o	o
		124. N. Y.	419	+	+	108. B. rutilescens	281. N. Y.	195	o	o
		125. J.H.U. (Isolated from feces)		+	+	109. B. pfeiffer	282. N. Y.	240	o	o
						110. B. acidi lactici	283. N. Y.	131	o	o
59. Streptococcus gracilis		126. N. Y.	128	+	+		284. N. Y.	135	o	o
		127. N. Y.	221	+	+		285. N. Y.	243	o	Delay o
		128. N. Y.	247	+	+	5 strains	286. N. Y.	446	o	o
60. Streptococcus lactis		129. N. Y.	254	+	+		287. N. Y.	447	o	+
61. M. rheumaticus, 3 strains		130. N. Y.	34	+	+	111. B. aquatilis	288. N. Y.	470	o	+
		131. N. Y.	259	+	+		289.			
		132. N. Y.	399	+	+	112. M. melitensis	290. N. Y.	33	?	+
62. Diplococcus (isolated from joint)		133. N. Y.	415	+	+	113. B. anthracis symptomatici,[8] 2 strains	290. N. Y.	48	?	+
							291. N. Y.	53	?	+
63. Diplococcus (isolated from cystitis)						114. B. edematis maligni[9]	292. N. Y.	485	?	+
		134. N. Y.	416	+	+	115. Diplococcus pneumoniæ, 2 strains	293. N. Y.	93	+	?
64. Mycobact.		135. Isolated at J.H.U. (V.)		+	+		294. Isolated from blood (Gatches)		+	o

	128. N. Y.	247	+	+		5 strains	286. N. Y.	446	◻	o
60. Streptococcus lactis	129. N. Y.	254	+	+			287. N. Y.	447	o	+
61. M. rheumaticus, 3 strains	130. N. Y.	34	+	+	111. B. aquatilis		288. N. Y.	470	o	+
	131. N. Y.	259	+	+			289.			
	132. N. Y.	399	+	+	112. M. melitensis		290. N. Y.	33	?	+
62. Diplococcus (isolated from joint)	133. N. Y.	415	+	+	113. B. anthracis symptomatici,[8] 2 strains		290. N. Y.	48	?	+
							291. N. Y.	53	?	+
63. Diplococcus (isolated from cystitis)	134. N. Y.	416	+	+	114. B. edematis maligni[9]		292. N. Y.	485	?	+
64. Mycobact. diphtheriæ	135. Isolated at J.H.U. (Y.)		+	+	115. Diplococcus pneumoniæ, 3 strains		293. N. Y.	93	+	?
	136. Isolated at J.H.U.	182	+	+			294. Isolated from blood (Gatches)		+	◻
	137. Isolated at J.H.U.	1157	+	+			295. Isolated from blood (Stewart)		+	Delay
	138. Isolated at J.H.U. (Clough)		+	+	116. B. lactimorbi		296. N. Y.	192	?	+
	139. Isolated at J.H.U.	356	+	+			297. N. Y.	189	o	?
	140. Isolated at J.H.U.	647	+	+		5 strains	298. N. Y.	190	o	?
	141. Isolated at J.H.U.	481	+	+			299. N. Y.	191	o	?
15 strains	142. Isolated at J.H.U.	148	+	+			300. N. Y.	188	+	+
	143. Isolated at J.H.U.	67	+	+	117. B. pyrogenes equinus		301. N. Y.	286	o	o
	144. Isolated at J.H.U.	711	+	+	118. Azoobacter. chroococcum		302. N. Y.	141	?	o
	145. Isolated at J.H.U.	131	+	+				252	?	+
	146. Isolated at J.H.U.	396	+	+	119. B. bulgaricus		303. N. Y.	67	+	o
	147. Isolated at J.H.U.	372	+	+				308	+	+
	148. Isolated at J.H.U.	404	+	+	120. B. oleraceæ, 2 strains		304. N. Y.	327	o	+
	149. N. Y.	100	+	?			305.	486	o	+
65. Mycobact. diphtheriæ columbarum	150. N. Y.	478	+	+	121. B. crensus		306. N. Y.	119	o	o
66. Mycobact. pseudo-diphtheriæ, 3 strains	151. N. Y.	2	+	+	122. B. proteus viridis		307. N. Y.	429	o	o
	152. N. Y.	21	+	+	123. B. proteus vulgaris 4 strains		308. N. Y.	142	o	o
	153. N. Y.	99	?	?			309. N. Y.	226	o	o
67. B. tetani, 2 strains	154. N. Y.	1	+	+			310. N. Y.	227	o	o
	155. N. Y.	274	+	+			311. J.H.U.		o	o
68. Spirillum rubrum	156. N. Y.	276	+	+	124. B. mirabilis,[10] 2 strains		312. N. Y.	411	o	o
69. Streptococcus endocarditidis 4 strains	157. Isolated from human blood (M.W.)		+	+			313.	489	+	+
					125. Enterococcus		314. N. Y.	64	?	o
	158. Isolated from human blood (Schnurr)		+	+	126. B. septicus murium		315. N. Y.	211	?	o
					127. B. melonis		316. N. Y.	305	?	o
	159. Isolated from human blood (Gaudron)		+	◻	128. B. vulgatus		317. N. Y.	362	?	o
					129. Spirillum milleri		318. N. Y.	441	o	o
	160. Isolated from human blood (Ladd)		+	o	130. B. cloacæ, 3 strains		319. N. Y.	23	o	o
							320. N. Y.	233	o	+
70. M. zymogenes	161. J.H.U.		+	Delay o			321. N. Y.	450	?	o
71. B. prodigiosus 4 strains	162. N. Y.	81	o	o	131. Diplococcus gonorrheæ, 2 strains		322. Torrey	0	o	Delay
	163. N. Y.	159	o	o			323.	L	o	+
	164. N. Y.	160	o	o	132. Mycobact. tuberculosis, 4 strains		324. N. Y.	87	+	+
	165. J.H.U.		o	o			325. N. Y.	257	+	o
72. B. pyocyaneus	166. N. Y.	102	o	o			326. N. Y.	267	+	+
	J.H.U.		o	o			327. Saranac		+	?
73. B. indicus 4 strains	167. N. Y.	186	o	o	133. Mycobact. moelleri, 2 strains		328. N. Y.	210	+	o
	168. N. Y.	231	o	o			329. N. Y.	213	+	o
	169. N. Y.	185	o	o	134. B. smegmatis		330. J.H.U.		+	Delay
	170. N. Y.	186	o	o	135. B. welchii		331. J.H.U.		+	o
74. B. violaceus, 2 strains	171. N. Y.	417	o	o	136. B. sporogenes		332. N. Y.	38	+	o
	172. J.H.U.		o	o	137. B. mallei, 2 strains		333. N. Y.	79	o	+
75. B. cyanogenes	173. N. Y.	352	o	o			334. N. Y.	269	o	+

[4] This specimen was definitely Gram positive.
[5] Stated by Matzuschita to be negative, but positive in the specimen studied.
[6] Stated by Matzuschita to be negative, but positive in the specimen studied.
[7] The specimen studied was completely decolorized, though the organism was described by Dawson as positive.
[8] Only partially decolorized; statements as to Gram stain vary.
[9] Partially decolorized.
[10] Apparently two different organisms.

EFFECT OF THE DYE TESTED BY ANIMAL INOCULATION.

These and other observations indicate clearly that gentian violet may exhibit its selective action in two ways. The dye is toxic for violet positive organisms when applied directly to them; and it is highly potent in preventing their development when incorporated in the media on which they are planted. For violet negative organisms it is not toxic; and in media it is without effect on their growth except in extremely strong dilutions. Its bacteriostatic[11] action on the violet positive organisms is evident enough. Has it any bactericidal power?

The cultural studies seemed to indicate that the organisms were usually killed. Fresh transplants from the surface of the agar on which the stained organisms had been stroked usually failed to grow, as did also transplants from the gentian violet side of a divided plate which had been inoculated with a violet negative organism. Nor was it usually possible to obtain growth by pouring broth into an agar tube which had been inoculated with stained organisms, and emulsifying the organisms on the surface of the agar with the broth, although this is an extremely effective method of reviving injured bacteria. At the same time, the occasional growth of organisms that had been stained, particularly the growth of organisms on media that were more favorable than plain agar, suggested the possibility that the bacteria were being simply restrained in their growth; and it was difficult in cultural experiments to eliminate the effect of the small amount of dye carried over by the oese into the media in making the transplant. The question was therefore tested by animal experimentation, three organisms being used for this purpose.

In the first experiments, a strain of blastomyces recently isolated from guinea pigs was employed. This was a particularly favorable organism for this purpose, because (1) cultural tests showed it to be violet positive; (2) it was constantly toxic for guinea pigs; (3) it produced characteristic lesions and was readily isolated from the heart's blood of the dead animals; and (4) though constantly toxic,

[11] This word was suggested by Professor Basil Gildersleeve. It indicates that the dye suspends reproduction, without implying that the organisms are necessarily killed.

it was not extremely so, could be used in large amounts, and caused death only after about three weeks.

Under ether anesthesia the guinea pigs were inoculated directly into the jugular vein with 1 c.c. of a thick emulsion of the blastomyces and with a second c.c. directly into the subcutaneous tissues of the neck. In the control animals, unstained organisms were used. The experimental animals received organisms which had been stained by adding 5 drops of a concentrated aqueous solution of gentian violet to 1 c.c. of emulsion and allowing this to stand for two hours. At the same time, strokes of the emulsion were made on divided plates (glucose agar), and inoculations of both stained and unstained organisms were made on glucose agar slants. In the first series, three animals were used. The results of the complete experiment are as follows:

Cultures.	1. Control glucose agar tube	Abundant growth.
	2. Tube inoculated with stained organisms	Sterile for several days; then a few scattered colonies.
	3. Divided plate	Luxuriant growth on plain agar; none on gentian violet agar.
Animals.	1. Control animal	Dead in 20 days; no lesions. Injected organism isolated in pure culture from heart's blood.
	2. Animal receiving stained organisms	Still alive; well (4 months after inoculation).
	3. Animal receiving stained organisms	Still alive; well (4 months after inoculation).

These experiments indicate that gentian violet applied directly to organisms may bring about their death, for when stained organisms were transplanted into the most favorable possible medium, and under conditions in which the inhibitive action of the dye (carried over at the time of the inoculation) could be excluded, they did not develop.

In the second series, five animals were used. The protocol follows.

Cultures.	1. Control glucose agar tube	Abundant growth.
	2. Tube inoculated with stained organisms	No growth.
	3. Divided plate	Abundant growth on plain agar; none on gentian violet agar (figure 12).

Animals.	1. Control animal	Dead in 11 days; characteristic lesions in spleen, from which injected organism was isolated in pure culture.
	2. Control animal	Dead in 17 days; no autopsy.[12]
	3. Animal injected with stained organisms	Still alive and well (3 months after inoculation).
	4. Animal injected with stained organisms	Still alive and well (3 months after inoculation).
	5. Animal injected with stained organisms	Dead 2 months after inoculation; no autopsy.[12]

A similar experiment was next done on rabbits with *Micrococcus aureus*. In the first series only two animals were used. One cubic centimeter of a broth culture was employed and was injected into the ear vein. The protocol follows.

Cultures.	1. Control agar tube	Abundant growth.
	2. Tube inoculated with stained organisms	No growth.
	3. Divided plate	Luxuriant growth on the plain agar; none on the gentian violet agar.

Animals.	1. Control rabbit	Dead in 24 hours. *Micrococcus aureus* was isolated in pure culture from the heart's blood.
	2. Rabbit receiving stained organisms	Still alive and well (3 months after inoculation).

These experiments proved that even enormous doses of a virulent staphylococcus might be rendered harmless by previous staining with gentian violet; for two animals which received such an intra-

[12] These two animals died during my absence from the city.

In the second series, six rabbits were inoculated, with the following results.

		48 hours.	72 hours.	4 days.	5 days.	7 days.	1 month.
Controls.	Rabbit 1.	Dead. *M. aureus* in pure culture from heart's blood.					
	Rabbit 2.	Alive.....	Dead. *M. aureus* in pure culture from heart's blood.				
Injected with stained organisms.	Rabbit 3.	Alive.....	Alive.....	Alive....	Dead. Blood cultures negative.		
	Rabbit 4.	Alive.....	Alive.....	Alive....	Dead. Blood cultures negative.		
	Rabbit 5.	Alive.....	Alive.....	Alive....	Alive....	Dead. Blood cultures negative.	
	Rabbit 6.	Alive.....	Alive.....	Alive....	Alive....	Alive....	Alive and well.

venous dose are alive and well a number of weeks after injections which, unstained, proved fatal to control animals in forty-eight to seventy-two hours. The delayed death in rabbits 3, 4, and 5 (experiment II), with the absence of the organisms in the circulating blood, raises a number of problems; but the fact that two rabbits survived the enormous dose, proves the efficiency of the dye.

The experiments with *B. anthracis* were done on colored mice, and the injections were made subcutaneously at the root of the tail. The organism used had previously been tested on divided plates, and by transplantation after staining; and had been found violet positive. In the first series the animals received $\frac{1}{3}$ c.c. of a broth culture.

		20 hours.	48 hours.	72 hours.	96 hours.
Controls.	Mouse 1..	Dead. *B. anthracis* in kidney, spleen, and blood			
	Mouse 2..	Alive.....	Dead. *B. anthracis* in kidney, spleen, and heart's blood.		
Injected with stained organisms.	Mouse 3..	Alive.....	Alive.....	Dead. All cultures sterile.	
	Mouse 4..	Alive.....	Alive.....	Alive.....	Dead. All cultures sterile.

In the second series, six mice were injected, the dose used being 0.02 c.c. of a broth culture. The results were as follows:

		24 hours.	48 hours.	72 hours.	5 days.	6 days.
Controls.	Mouse 1..	Dead. *B. anthracis* in heart's blood.				
Controls.	Mouse 2..	Dead. *B. anthracis* in heart's blood.				
Controls.	Mouse 3..	Alive.....	Dead. *B. anthracis* in heart's blood.			
Injected with stained organisms.	Mouse 4..	Dead. *B. anthracis* in heart's blood.				
Injected with stained organisms.	Mouse 5..	Alive.....	Dead. *B. anthracis* in heart's blood.			
Injected with stained organisms.	Mouse 6..	Alive.....	Alive.....	Alive....	Alive....	Dead. All cultures negative.

The protocol for the third series, in which six mice were injected with 0.02 c.c. of a broth culture, follows.

		24 hours.	36 hours.	3 days.	1 week.	9 days.	10 days.
Controls.	Mouse 1..	Alive....	Dead. *B. anthracis* in spleen and heart's blood.				
Controls.	Mouse 2..	Alive....	Dead. *B. anthracis* in spleen and heart's blood.				
Injected with stained organisms.	Mouse 3..	Alive....	Dead. *B. anthracis* in spleen and heart's blood.				
Injected with stained organisms.	Mouse 4..	Alive....	Dead. *B. anthracis* in spleen and heart's blood.				
Injected with stained organisms.	Mouse 5..	Alive....	Dead. *B. anthracis* in spleen and heart's blood.				
Injected with stained organisms.	Mouse 6..	Alive....	Alive.....	Alive..	Alive..	Alive..	Dead. A few organisms in smears from spleen and blood. All cultures negative.

There are several features in these experiments with *Bacillus anthracis* which cannot at present be explained; and certain questions are raised by them which will be the subject of a subsequent report. For present purposes, the important fact is, that while no animal injected with stained organisms survived, in several life was much prolonged. We were working with enormous doses of an organism which killed the control animals in about thirty-six hours. Yet one animal, injected with stained organisms, lived six days; and another died on the tenth day. Probably in these instances a few individual vegetative cells or spores escaped death, but were so injured by the dye that growth was greatly retarded.

EFFECT OF THE DYE ON SPORES.

It is manifest, from what has been said, that the spores are at least gravely affected by treatment with the dye; and that, in the presence of gentian violet, they cannot develop; or, if they become vegetative cells, their growth ceases at that stage.

Are they actually killed? In order to test this point the old silk thread experiments of Koch were done.

Silk threads were soaked in broth cultures of *B. subtilis* that had grown for one day and for ten days and were then immersed in a saturated aqueous solution of gentian violet for one hour. As much of the stain as possible was washed out in sterile water, and the threads were dropped into broth tubes. With a very few exceptions, these tubes remained sterile; while the controls, of course, always showed abundant growth within twenty-four hours.

From these and the other cultural studies it appears that even the spores may be killed by this dye,—a fact altogether astonishing in view of the failure of the spores to take the stain. The occasional growth, however, of inoculations of stained spore-bearing organisms, and the results of the animal experiments with *Bacillus anthracis,* indicate that the action of the dye on spores is not absolutely constant, and that certain " fast " individuals may escape.

SUMMARY OF THE EFFECT OF STAINING.

As far as the effect of gentian violet on bacteria is concerned, the cultural and animal studies warrant the following statements.

1. The growth of violet positive organisms is usually completely

inhibited, provided the stained organisms are transplanted to agar. When they are transplanted into broth, the results are similar, but are less constant.

2. The spores of the gentian violet organisms are also gravely affected by the dye, and practically never grow when transplanted to agar; though they occasionally grow when transplanted into broth or when injected into the animal body.

3. The violet positive organisms may be so injured by staining with the dye that they do not develop even in the most favorable environment within the animal body.

4. Occasionally the stained organisms grow in the animal body, but their growth is greatly retarded.

5. It is probable that an occasional "fast" individual (either vegetative cell or spore) escapes entirely the effect of the dye.

6. The effect of gentian violet on violet positive organisms is better described as bacteriostatic, rather than as bactericidal. Indeed, the term bacteriostatic, may be applied accurately to many "bactericides," for most of these bring about the death of the organisms only under certain conditions of intensity and duration of application.

QUESTION OF VITAL STAINING.

It seems probable that the selective action which gentian violet exhibits when it is added to broth cultures of *Bacillus subtilis* or *Bacillus prodigiosus* depends on the capacity of the dye to stain the organisms during life. If hanging drops of these bacteria be made, and a small amount of gentian violet be added, it will be noted that *Bacillus subtilis* takes up the stain rapidly and intensely, and that the actively motile individuals soon become quiet. This applies to most of the organisms seen, although not all are stained with equal promptness or intensity, and a few stain very slowly indeed. *Bacillus prodigiosus,* on the other hand, behaves quite differently. Here staining proceeds much more slowly, never becomes so intense, and motility is only slightly influenced. The conclusion that vital staining really takes place is based on the fact that organisms have been observed which take up the dye, but remain actively motile.

EFFECT OF FIXATION OF THE DYE.

Several attempts have been made to fix the dye in the bacteria, in order to determine whether such a fixation would render the gentian violet toxic even for the Gram negative organisms.

In the first few experiments, Gram's reagent was used as the fixative; and the organisms were transplanted to agar after vitally staining and treating them with the iodin solution. It was found, however, by control experiments that the Gram reagent alone killed the bacteria; so that ammonium molybdate was substituted as a fixative. To 1 c.c. of a broth culture of *B. coli,* five drops of concentrated aqueous gentian violet were added, and the mixture was allowed to stand for one hour. It was then centrifugalized until the stained organisms were at the bottom of the tube, the supernatant liquid was poured off, ammoniun molybdate was added and the mixture was well shaken. Then, after the mixture had stood for ten minutes, it was again centrifugalized and stroke inoculations were made on agar from the organisms at the bottom of the tube. So far as could be determined growth was not impeded.

CAUSE OF THE SELECTIVE ACTION OF THE DYE.

No real light has as yet been thrown on the intimate nature of this selective action of the dye. It does not depend on the presence of arsenic (one of the commoner impurities of aniline dyes), for in the analyses[13] no trace of arsenic could be detected. Nor does it depend on any change in the reaction of the media, for even in fairly strong solutions gentian violet is so nearly neutral that its slight alkalinity can be detected only by methods which are exceedingly delicate; and in the dilutions in which it occurred in the media used in these experiments, it may be regarded as absolutely neutral. Filtration of saturated aqueous gentian violet through charcoal will remove every visible trace of the stain, and the filtrate has absolutely no bactericidal power.

The possibility that this selective property depended on some light phenomenon was investigated. Divided plates, inoculated with *Bacillus subtilis* and *Bacillus prodigiosus* and kept in absolute darkness, showed the same results as when the organisms were grown in the light.

The dye was also examined for the possession of radiant properties; and on one photographic plate, subjected to its influence in absolute darkness, a fairly good picture of a silver coin was ob-

[13] The analyses were made by Dr. Walter Jones.

tained. It seemed probable that this phenomenon was due to a chemical emanation similar to that described by Russell,[14] as given off by wood, varnish, etc., and capable of affecting a photographic plate in the dark. The subsequent experiments in the case of gentian violet were not particularly convincing.

QUANTITATIVE CONSIDERATIONS.

The evidence of numerous experiments made to investigate this point shows that the bacteriostatic action of gentian violet is, like the action of all bactericides, fundamentally a quantitative one; that is to say, when the dye is applied in sufficient intensity and for a sufficiently long time, it will probably inhibit or kill all organisms. The interval, however, between the dilution necessary to prevent the growth of *Bacillus subtilis* completely, and that required to have an appreciable effect on *Bacillus prodigiosus,* is so wide that the selective action may almost be thought of as independent of any quantitative element. Notice, for example, the results of the following experiment, which was made repeatedly.

Four Gram positive organisms were planted in broth tubes containing gentian violet in a dilution of 1:1,000,000; and at the same time control inoculations were made in plain broth. In none of the violet broth tubes did growth occur, as was tested by inoculations made from them at varying intervals over a period of several days. The results of this experiment are shown in the following table.

Dilution 1: 1,000,000.

		48 hours.	6 days.	9 days.	13 days.	Controls.
Gentian violet broth	1. *B. subtilis*....	0	0	0	0	+
	2. *M. aureus*....	0	0	0	0	+
	3. *M. albus*.....	0	0	0	0	+
	4. *B. diphtheriæ*.	0	0	0	0	+

Agar Slants from Violet Broth Tubes after Forty-Eight Hours' Incubation.

		48 hours.	5 days.
48 hour agar slants	1. *B. subtilis*.....	0	0
	2. *M. aureus*.....	0	0
	3. *M. albus*.....	0	0
	4. *B. diphtheriæ*.....	0	0

[14] Russell, W. J., *Proc. Roy. Soc.,* 1906, *Series* B, lxxxviii, 385; *Phil. Tr. Roy. Soc.,* 1904, *Series* B, cxcvii, 281.

Agar Slants from Violet Broth Tubes after Six Days' Incubation.

		48 hours.	6 days.
6 day agar slants	1. *B. subtilis*	0	0
	2. *M. aureus*	0	0
	3. *M. albus*	0	0
	4. *B. diphtheriæ*	0	0

Agar Slants from Violet Broth Tubes after Thirteen Days' Incubation.

		5 days.
13 day agar slants	1. *B. subtilis*	0
	2. *M. aureus*	0
	3. *M. albus*	0
	4. *B. diphtheriæ*	0

A dilution of 1 to 1,000,000 is thus seen to stop the growth of these organisms; and their growth may be retarded even by a dilution of 1 to 2,000,000. With these weak dilutions, however, the results are not absolutely constant; growth is usually delayed, but may proceed slowly. In media in strengths of 1 to 100,000, however, the dye is quite efficient. The violet positive organisms never grow on the surface of agar containing the dye in this quantity. On the other hand, the violet negative organisms are uninfluenced by a dilution of 1 to 100,000. They grow readily in a dilution of 1 to 10,000, irregularly in one of 1 to 5,000, and often in one of 1 to 1,000. For the separation, therefore, of the violet positive from the violet negative organisms, dilutions of 1 to 100,000 or 1 to 75,000 are efficient. This strength is well above that necessary to check the violet positives and well below that likely to restrain the violet negatives. Nor is it at all necessary, for practical purposes, that this dilution be accurately made.

I have found that one loopful of concentrated aqueous gentian violet, measured with an oese 2 mm. in diameter, added to 7 c.c. of broth gives a dilution of approximately 1:70,000. For practical purposes, one may make up a tube in which the dilution is accurately measured and, using that as a standard, make subsequent dilutions colorimetrically as gauged by the eye. All that is usually necessary, however, is to add to an ordinary tube of media an ordinary loopful of concentrated aqueous gentian violet. The eye soon learns to tell whether too much or too little has been added; and for practical purposes this method is sufficiently accurate. It should perhaps be stated that the results recorded in this paper were obtained with dilutions of 1:100,000. This was the dilution of the gentian violet in figures 5 and 6.

The effect of the dye when applied directly to the bacteria varies with the duration of its application.

For *B. subtilis,* one hour's staining with gentian violet in the strength of four drops of dye to 1 c.c. of a forty-eight hour culture is usually sufficient to prevent growth, when the organisms are transplanted to agar. Often a much shorter time suffices. An absolute correspondence between the effect of the dye and the duration of its application to the organisms could not be established.

For *B. prodigiosus* and *B. lactis aerogenes,* on the other hand, staining for hours is without effect. In experiments made to test this point, a mixture of *B. typhosus, B. vulgaris, B. subtilis, B. prodigiosus, B. pyocyaneus, B. coli,* and *B. lactis aerogenes* was treated with gentian violet, and plates were made after 24, 48, 72, and 96 hours. In the early plates, abundant mixed growth occurred; in the 72 hour plates, only *B. prodigiosus* and *B. lactis aerogenes* could be distinguished; and these two organisms were still present in plates made from the specimens which had been stained for 96 hours.

STUDY OF MIXED CULTURES.

The application of the selective action of gentian violet in the study of mixed cultures proved highly interesting; and it has been abundantly shown that the use of the dye in divided plates may provide assistance of some importance in practical bacteriology.

The earliest experiment of this sort was concerned with a mixture of *B. subtilis* and *B. prodigiosus;* and it was found that a pure culture of the latter organism could easily be obtained by adding to the mixture a few drops of gentian violet, and allowing it to stand for an hour. Then, upon transplanting the mixture to agar, *B. subtilis* disappeared and *B. prodigiosus* grew out in pure culture.

A similar experiment was also done with a culture of a luxuriantly growing, acid-fast organism (*B. tuberculosis ?*), which had become contaminated with *B. subtilis.* The contamination was readily eliminated by staining the mixture with gentian violet; the original organism was unaffected and grew out in pure culture.

Similar isolation of violet negative organisms from a mixture with violet positive organisms was readily accomplished by growing the mixture in broth to which gentian violet had been added. The following experiment may be cited as an example.

A Gram negative organism (*B. typhosus*) was added to a mixture of Gram positive organisms (*M. aureus, M. albus, B. subtilis, B. diphtheriæ, Streptothrix actinomyces,* and *B. anthracis*), and grown in gentian violet broth. Transplants were then made on agar and the Gram negative organism grew out in pure culture, the growth of the Gram positive organisms being completely inhibited.

A similar result was obtained when the Gram negative organisms employed were *B. prodigiosus, B. pyocyaneus,* or *B. lactis aerogenes.*

This isolation in pure culture was accomplished in the easiest and most clear cut way by the use of divided plates. Strokes were made on divided plates

from broth cultures containing a number of different organisms, or from a salt solution emulsion of several different organisms which had been grown on agar. The Gram positive organisms grew out on the plain agar side, but stopped some distance from the dividing line, and from that point the Gram negative organism grew in pure culture.

In figure 13, for instance, three stroke cultures were made of a mixture of violet positive organisms (*B. subtilis,* two strains of *M. aureus, M. albus,* two strains of *B. diphtheriæ*), and one violet negative organism (*B. typhosus*). It will be seen that the growth of the violet positives ceases about 1 cm. from the dividing line; and from that point *B. typhosus* has grown out in pure culture across the gentian violet agar.

In figure 14 the effect of the gentian violet is even more strikingly shown. On the left hand side of the plate a mixture of *B. subtilis* and *M. aureus* had been stroked, but no growth whatever occurred on the gentian violet agar. On the right hand side of the plate, the same mixture plus *B. typhosus* had been planted, and a pure culture of *B. typhosus* was found on the gentian violet agar. This experiment and similar ones in which other violet negative organisms were isolated in purity were repeatedly carried out. The constancy of the results and their clear cut character demonstrated how easy it was to obtain pure cultures by this method.

Fortunately, almost all the air contaminations (*Bacillus subtilis,* cocci, yeasts) are violet positive, and will not grow in the presence of the dye. This fact is illustrated in figure 15, which represents a divided plate that had been exposed before an open window for one hour on a windy day. Numerous colonies, it will be seen, are growing on the plain agar; none whatever on the gentian violet side.

It is thus the simplest matter in the world to free from air contaminations a culture of a violet negative organism. All that is necessary is to grow it for twenty-four hours in gentian violet broth, or to stroke it on a divided plate. Inasmuch as a number of the organisms used in animal experimentation are violet negative *Bacillus prodigiosus, Bacillus coli,* etc.), and it is in animal experimentation that contaminations are frequent, this method of getting rid of contamination ought to be of value.

RECOGNITION OF CONTAMINATIONS.

That certain cultures supposed to be pure are really mixed has been demonstrated with striking ease by the gentian violet divided plate method.

During the study of the stock cultures in the New York Museum, No. 106, a culture of *B. subtilis* (isolated by Dawson from the soil) grew on a divided

plate as shown in figure 16. In the great number of transplants of *B. subtilis* previously made, growth on the gentian violet side had never occurred; and the appearance of this plate, as will be seen from the illustration, indicated pretty strongly that two organisms were present. Smears showed the organism growing on the plain agar side to be a definitely Gram positive, spore-bearing bacillus (*B. subtilis,* figure 17). Smears from the gentian violet side revealed a short bacillus (without spores) which completely decolorized by the Gram method (figure 18). This organism had the following characteristics:

Motility	Sluggish.
Gram stain	Completely decolorized.
Spores	None.
Glucose	Gas produced.
Milk	Acidified, clotted, no peptonization.
Gelatin	No liquefaction.

We were dealing, evidently, with a mixed culture of *Bacillus subtilis* and *Bacillus coli,* as the divided plate readily revealed. This contamination would not, I think, have been suspected in the ordinary laboratory routine; for on all ordinary transplants *Bacillus subtilis* promptly outgrew the *Bacillus coli.* Indeed, in this instance, it had not been suspected.

More recently I have had occasion to make a similar observation with *Bacillus diphtheriæ.*

This organism, in numerous transplants, had always refused to grow on the gentian violet agar, stopping some distance from the dividing line. No. 481, however, isolated in a recent study of a diphtheria epidemic and supposed to be a pure culture, grew across the plate just as a violet negative organism does. Cultures were, therefore, made from each side of this plate, and these revealed that we were dealing with a mixed culture of *B. diphtheriæ.* Each of the three organisms present was then isolated in pure culture; the *B. diphtheriæ* proved virulent for animals, and the three organisms were again planted on divided plates. *B. diphtheriæ,* now in pure culture, failed to grow out on the gentian violet side; *i. e.,* it behaved as all previous strains had done. The contaminating organisms, on the other hand, grew in equal luxuriance on the two sides. This is well shown in figure 19 which is from a plate on which the two contaminating organisms, a known pure culture of *B. diphtheriæ,* and a pure culture of No. 481 were planted. It will be seen that *B. diphtheriæ* refused to grow on the gentian violet agar, while the contaminating organisms grew across the whole plate.

SEPARATION OF CLOSELY ALLIED ORGANISMS.

At first it was not thought likely that this method would be applicable to the separation of closely related organisms, as these might be expected to react in the same way to the dye. My ex-

perience, however, with one group of very closely allied bacteria shows how extremely specific and sensitive this reaction is.

In this instance, five strains, indistinguishable from each other by the ordinary cultural, morphological, or tinctorial tests, were planted on divided plates. Four of these grew across with constancy. With equal constancy one refused to grow in the presence of the gentian violet.

These organisms belong in the *Bacillus enteritidis* group and, though they have not been agglutinated with known serum, they are referred to in the table as *Bacillus enteritidis,* because the stock cultures from which they came were so named. For present purposes, it is a matter of indifference whether they are true to name or not; the important point is that of five organisms (New York, 18, 25, 26, 132, and 234) identical in all ordinary cultural and staining reactions, four were constantly violet negative and the fifth constantly violet positive (figure 20). The characteristics of these organisms were as follows:

	No. 18.	No. 25.	No. 26.	No. 132.	No. 234.
Morphology	Short bacilli; no spores.	Short bacilli; no spores.	Short bacilli; no spores.	Short bacilli; no spores.	Short bacilli; no spores.
Motility	+	+	+	+	+
Gram stain	o	o	o	o	o
Milk	Slight initial acidity; no coagulation.	Slight initial acidity; no coagulation.	Slight initial acidity; no coagulation.	Slight initial acidity; no coagulation.	Slight initial acidity; no coagulation.
Gelatine	No liquefaction.	No liquefaction.	No liquefaction.	No liquefaction.	No liquefaction.
Glucose	Fermented.	Fermented.	Fermented.	Fermented.	Fermented.
Lactose	Not fermented.	Not fermented.	Not fermented.	Not fermented.	Not fermented.
Saccharose	Not fermented.	Not fermented.	Not fermented.	Not fermented.	Not fermented.
Mannite	Fermented.	Fermented.	Fermented.	Fermented.	Fermented.
Gentian violet	o	o	o	o	+

That this exceptional behavior on the part of No. 234 was not a temporary freak, was proved by its constancy when transplanted from the Johns Hopkins stock culture and from the New York stock culture, which had originally come from the same source. The reaction is a constant and fundamental biological characteristic of this strain, which in all other respects is indistinguishable from the other four. This group of organisms is at present under study

as to agglutinative properties, and will be made the basis of a subsequent report.

PRACTICAL APPLICATIONS.

The observations here reported have the following practical applications to bacteriology.

1. The reaction of an organism to gentian violet, when planted on a divided plate, is a characteristic as easy to determine, as constant, as clear cut, and as significant as any known mark by which bacteria are identified. The Gram stain is not to be compared with it as regards definiteness or constancy. It fails to be of value in the case of the small number of organisms (*e. g.*, pneumococcus, *Diplococcus gonorrheæ*) which will not grow well on plain agar, or whose slight growth is inhibited by almost any change in the media. For a small group of organisms the reaction is not absolutely constant and therefore, in their case, not altogether satisfactory.

2. The isolation of violet negative organisms from mixtures with those which are violet positive is readily accomplished by transplantation on divided plates.

3. In the same way, cultures of violet negative organisms may readily be freed of air contaminations.

4. The transplantation on divided plates may be of assistance in demonstrating the presence of a contamination in a supposedly pure culture.

FURTHER PROBLEMS.

A number of problems for investigation are suggested by this work.

1. The effect of the dye on organisms not actually killed by it, *e. g.*, upon certain surviving cells of a stained culture of *Bacillus anthracis.*

2. Systematic investigation of other dyes, for which the technique of divided plates offers a simple method.

3. Extension of the method and use of other dyes in the attempt to obtain pure cultures out of mixtures. The *Treponema pallidum* particularly should be studied in this way. My own experiments on this point have not, as yet, been conclusive; but I have found that smears from genital sores, made on gentian violet agar, remained

sterile, while numerous contaminating organisms grew out on the control tubes.

4. The experience with the enteritidis group indicates how extremely specific the selective action of this dye is, and suggests that some of the vexed problems in the cultivation of closely related species may be similarly solved.

5. Growth of tissues in stained media, to see if a similar selective action could be brought to light.

6. The effect of staining on ultra-microscopical organisms. This investigation I have recently begun.

7. The effect of staining on inoculable tumors and their filterable virus.

8. The possible application of these observations to clinical infections. This problem is at present being studied in the laboratory of Yale University by both clinical and experimental methods.

HISTORICAL.

These observations on the selective action of the dye are, so far as I am aware, new; but that some of the aniline dyes are capable of injuring certain cells has long been known. Early observations of this sort were made by botanists on plant cells; and Cornil and Babes,[15] working with methyl violet, found that it possessed a similar toxic effect for bacteria. In 1890 Stilling[16] of Strassburg published an extensive thesis on this subject. His work was done with a dye (probably a mixture of dyes) which he called, generically, pyoktanin. His experiments were none too carefully performed with *B. subtilis, B. anthracis, M. aureus,* etc., but led him to the conclusion that the dye was a general bacterial poison, its antiseptic strength being "three times that of bichloride of mercury." He thought that the action of the dye was directly proportional to the strength of the dilution used and to the time of exposure; and that the cell membrane of the bacteria played an important part in the process. He failed to observe any selective action exhibited by the dye. On the basis of his poorly controlled observations, he introduced the dye therapeutically, making for it extravagant claims which Garré and Troje[17] were easily able to disprove. Other observers agreed with Stilling; but the choice of clinical infections, on which the drug was tried, was not wisely made, and the dye fell into disrepute.

In the literature since that time, scattered observations have been found on the action of many of the dyes on different species of organisms. None of

[15] Cornil and Babes, cited by Stilling, J., Anilin Farbstoffe als Antiseptica und ihre Anwendung in der Praxis. 1. Mittheilung. Strassburg, 1890.

[16] Stilling, J., *loc. cit.*

[17] Garré and Troje, *München. med. Wchnschr.,* 1890, xxxvii, 431.

the statements that have to do with characteristic changes produced in the stain by the organisms (Spina,[18] Noegerrath,[19] von Rosahegyi,[20] etc.) are very convincing; but a number of observers have noticed a definite bactericidal action possessed by various stains (Penzoldt,[21] von Rosahegyi, Krieger, etc.). Gentian violet, however, has received practically no notice from these observers.

The best known observation similar to ours is that of Drigalski and Conradi.[22] These authors worked with crystal violet, which they found inhibited the growth of numerous cocci and bacteria, though it was without influence on *B. typhosus* and *B. coli.* "A number of organisms belonging to the subtilis group" are mentioned among those that grew out on their crystal violet plates. Their chief interest was in the cultivation of *B. typhosus;* and the important lead as to the selective property of crystal violet was not followed up by a systematic study of the dye.

A similar lead was also obtained by Vay;[23] but his chief interest was in an investigation of the finer structure of bacteria; and the selective power of the dyes with which he worked (dahlia, methyl violet, and malachite green) was not studied.

EXPLANATION OF PLATES.

Plate 21.

Fig. 1. The tube on the left was inoculated with *B. prodigiosus* for control; that on the right with *B. prodigiosus* which had been stained for one hour with gentian violet. Note that the growth of the stained organisms was unaffected by the dye. Compare with figures 2 and 3.

Fig. 2. The tube on the left was inoculated with *B. subtilis* for control; that on the right with *B. subtilis* which was stained for one hour with gentian violet. Note that the growth of *B. subtilis* was completely inhibited by the dye. Compare with figures 1 and 3.

Plate 22.

Fig. 3. The tube on the left was inoculated with blastomyces for control; that on the right with blastomyces which had been stained for one hour with gentian violet. Notice that the growth of the stained organisms was completely inhibited by the dye. Compare with figures 1 and 2.

Fig. 4. The plate on the left was made from a broth tube which had been inoculated twenty-four hours before with *B. subtilis,* which had been stained with gentian violet. That on the right was made from a broth tube of *B. prodigiosus* similarly treated. Note that the *B. subtilis* plate is sterile, but that the *B. prodigiosus* plate is crowded with colonies.

[18] Spina, A., *Centralbl. f. Bakteriol., Orig.,* 1887, ii, 71.
[19] Noegerrath, E., *Fortschr. d. Med.,* 1888, vi, 1.
[20] von Rosahegyi, A., *Centralbl. f. Bakteriol., Orig.,* 1887, ii, 418.
[21] Penzoldt, F., *Arch. f. exper. Path. u. Pharmakol.,* 1890, xxvi, 310.
[22] Drigalski, V., and Conradi, H., *Ztschr. f. Hyg.,* 1902, xxxix, 283.
[23] Vay, F., *Centralbl. f. Bakteriol., Orig.,* 1910, lv, 193.

PLATE 23.

FIG. 5. The plate has been stroked with an emulsion of *Sarcina rosea*. The growth refuses to approach the dividing line. Note the mark of the inoculating needle in the gentian violet agar. The dye in the gentian violet agar is in the strength of 1: 100,000. Notice the diffusion of the stain into the plain agar, across the dead line.

PLATE 24.

FIG. 6. The plate has been stroked with an emulsion of *B. prodigiosus*. Growth has been uninfluenced by the dye. Note the staining of the growth on the gentian violet half of the plate.

PLATE 25.

FIG. 7. Two stroke cultures of *B. prodigiosus* have been made on this plate, separated by a stroke culture of *B. subtilis*, the selective action of the gentian violet being thus clearly demonstrated. *B. subtilis* refuses even to approach the gentian violet half of the plate; while *B. prodigiosus* grows equally well on the two sides of the dividing line.

FIG. 8. The plate has been stroked with *M. aureus*, flanked on either side by *B. typhosus*. The former organism ceases to grow at a point some distance from the dividing line; while the latter grows equally well on the two halves of the plate. The mark of the needle can be plainly seen, where the stroke culture of *M. aureus* was made in the gentian violet agar; but no growth whatever has taken place there.

PLATE 26.

FIG. 9. The plate has been stroked with three different strains of *B. subtilis*.

FIG. 10. The plate has been stroked with two different strains of *B. mycoides* (New York, 115 and 249), both of which refuse to grow in the presence of gentian violet.

PLATE 27.

FIG. 11. The plate has been stroked with three different strains of *B. anthracis* (New York, 107, 108, 109). The growth ceases sharply some distance from the dividing line, and none whatever has taken place on the gentian violet side.

FIG. 12. Two stroke cultures of blastomyces have been made. In the first week after inoculation the organism, which grows luxuriantly on the plain agar side, refuses to approach the dividing line. Later, however, the edge often advances somewhat and the growth may even cross for a few millimeters into the gentian violet half of the plate, but it never invades further.

PLATE 28.

FIG. 13. The plate has been stroked with a mixture of several Gram positive organisms (*B. subtilis*, two strains of *M. aureus*, *M. albus*, two strains of *B. diphtheriæ*) and one Gram negative organism (*B. typhosus*). Starting at the point, nearly 1 cm. from the dividing line, where the sharp margin indicates that the growth of the Gram positive organisms has ceased, *B. typhosus* has grown out in pure culture, extending in luxuriance across the gentian violet agar.

FIG. 14. The left half of the plate has been stroked with a mixture of *B. subtilis* and *M. aureus*. The luxuriant growth in the plain agar ceases sharply some distance from the dividing line; and the edge of the growth is seen to be stained by the dye which has diffused across the line. On the gentian violet agar there has been no growth whatever. The right half of the plate has been stroked with the same mixture plus *B. typhosus;* and at the point where the growth of *B. subtilis* and *M. aureus* ceases, a pure culture of *B. typhosus* is seen to start to cross the line, and to grow well on the gentian violet agar.

PLATE 29.

FIG. 15. This plate was exposed for one hour before an open window, on a windy day.

FIG. 16. The plate was stroked with a culture of *B. subtilis* (New York, 106) that was supposed to be pure. This organism grew luxuriantly on the plain agar side in its customary way, refusing as usual to grow on, or even to approach the gentian violet agar. From the point where the luxurious growth of *B. subtilis* ceased, another growth, obviously of a different organism, crossed over into the gentian violet half of the plate. This second organism was shown to be *B. coli.* Microphotographs of smears from the two sides are shown in figures 17 and 18. Figure 16 illustrates the isolation of an organism in pure culture from a mixture and shows the value of the method as an aid in determining whether or not a culture is pure.

PLATE 30.

FIG. 17. A smear made from the plain agar side of the plate shown in figure 16.

FIG. 18. A smear made from the gentian violet agar side of the plate shown in figure 16.

FIG. 19. A is the stroke made from an organism contaminating a growth of *B. diphtheriæ;* D, the stroke made from an organism contaminating a growth of *B. diphtheriæ;* C, the stroke made from a known pure culture of *B. diphtheriæ;* B, the stroke made from culture 481 (pure *B. diphtheriæ*). Note that, on the gentian violet side, the site of the original stroke (which was made from a thick emulsion of bacteria) is faintly to be seen. There has, however, been no apparent growth. It is possible that a few living organisms are present, though repeated transplants on Loeffler's serum remained sterile.

PLATE 31.

FIG. 20. On this plate five strains of *B. enteritidis* have been planted. All except 234 (on the extreme left) have grown in the gentian violet agar.

FIG. 1.

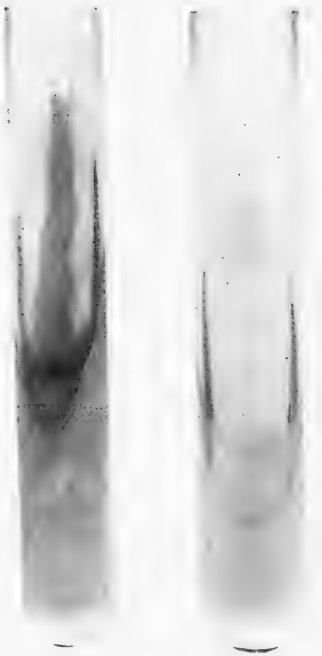

FIG. 2.

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FIG. 3.

FIG. 4.

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FIG. 5.

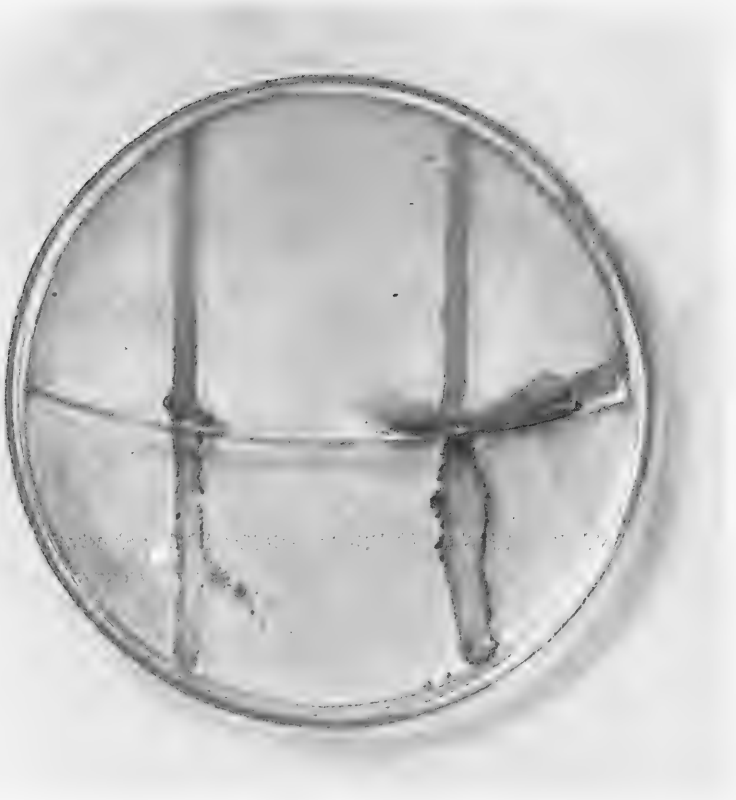

FIG. 6.

OF

FIG. 7.

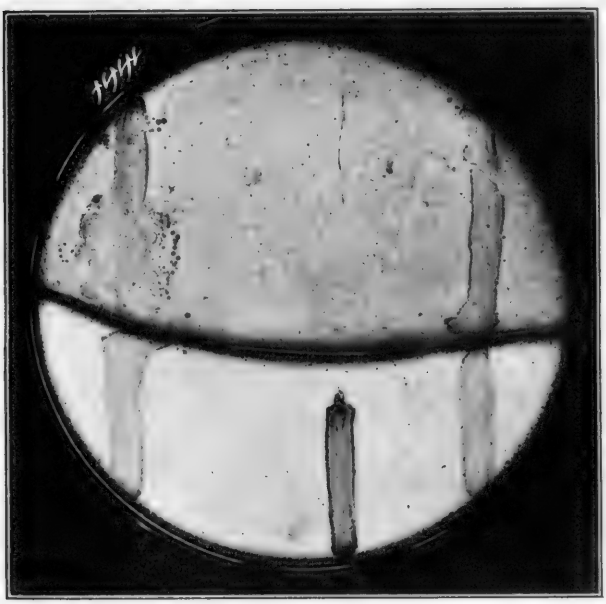

FIG. 8.

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FIG. 9.

FIG. 10.

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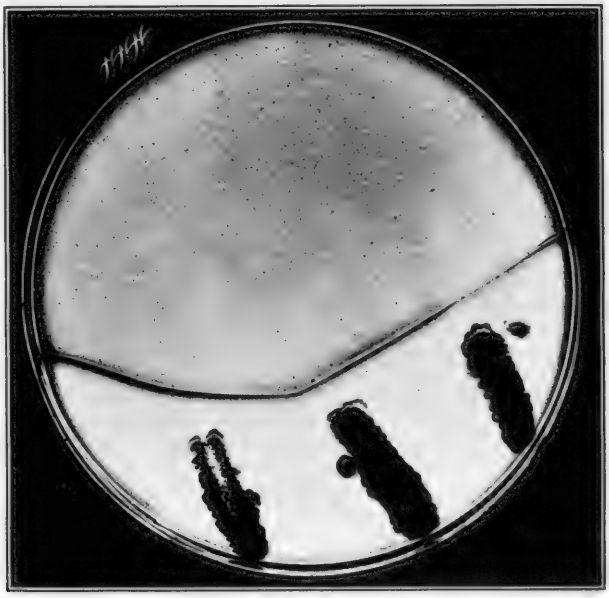

FIG. 11.

FIG. 12.

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FIG. 13.

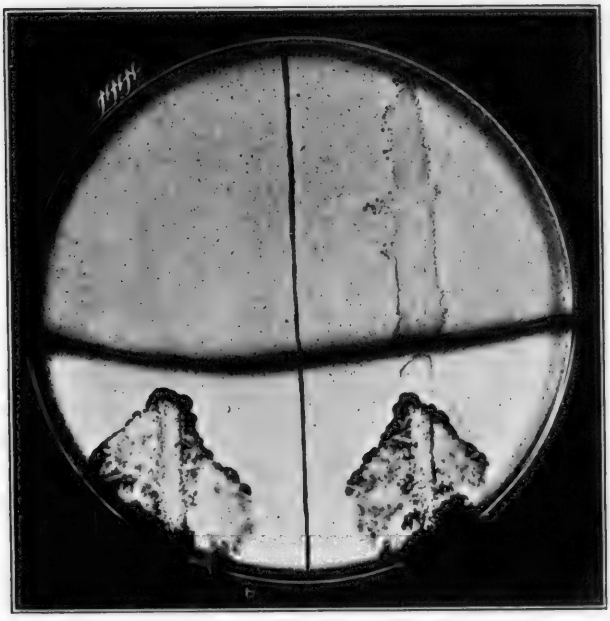

FIG. 14.

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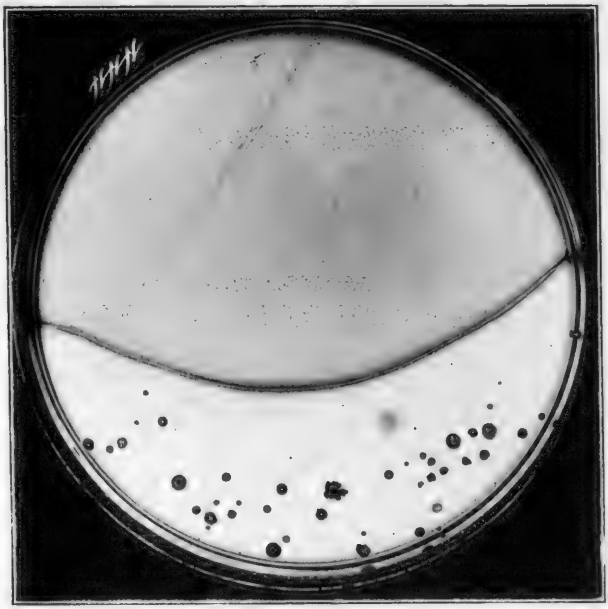

FIG. 15.

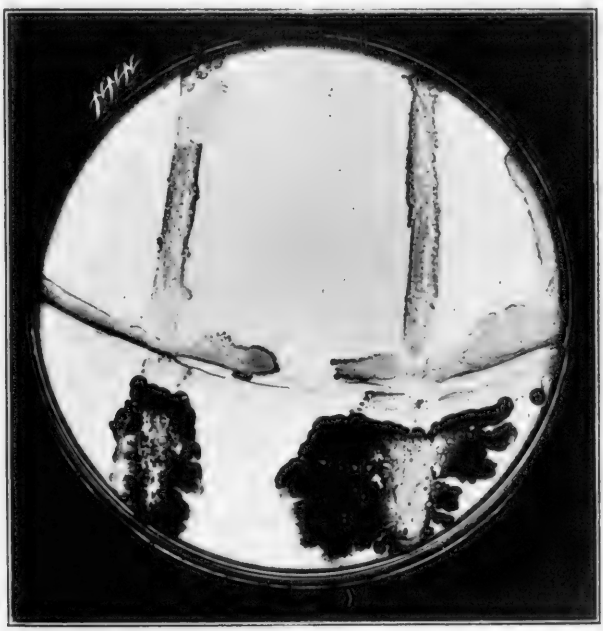

FIG. 16.

FIG. 17.

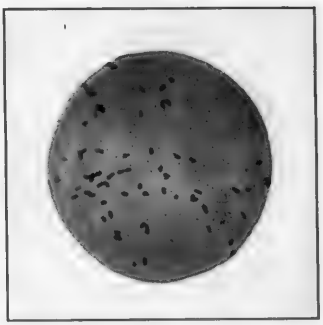

FIG. 18.

FIG. 19.

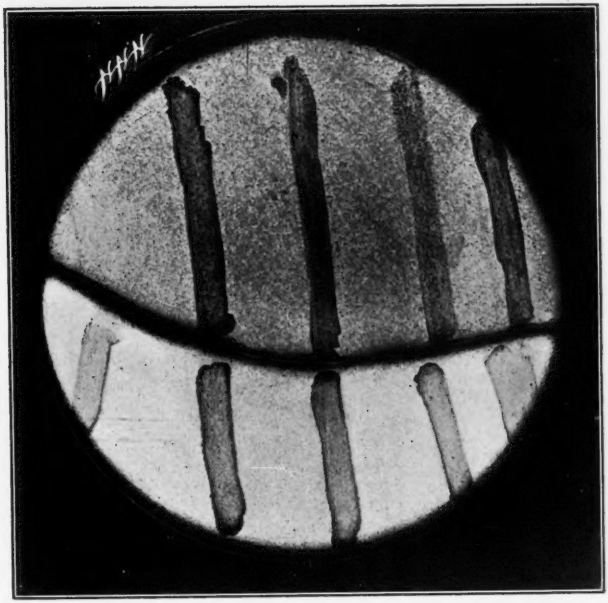

FIG. 20.

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